EXERCISE INDUCED DAMAGE TO SKELETAL MUSCLE AND CONNECTIVE TISSUE.

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Summary

Indices of human skeletal muscle damage and connective tissue breakdown were studied following eccentric and concentric muscle contractions of the knee extensors (KE).

Electrically stimulated eccentric contractions of the KE induced delayed onset muscle soreness (DOMS), a delayed increase in serum creatine kinase (CK) activity, and disruption of muscle function. KE maximum isometric contraction force (MVC), MVC with superimposed myostimulation, and muscle force-frequency characteristics (20:100 Hz stimulated force ratio) were impaired for up to 3 days post exercise. Increased delays in excitation-contraction (E-C) coupling were observed immediately post exercise and on day 3 post exercise. Unaltered contraction and relaxation kinetics suggested that the sarcoplasmic reticulum was not the site of E-C coupling delays.

Prior 'conditioning' eccentric exercise bouts of varying duration were used to examine skeletal muscle adaptation to a subsequent bout of 50 eccentric repetitions. All initial bouts (10, 30, and 50 repetitions) induced DOMS, a decline in MVC, and reduced 20:100 Hz stimulated force ratio. Initial bouts of 30 and 50 repetitions elevated serum CK activity (peak activities recorded on day 3 post exercise). The response of these indirect indices of muscle damage appeared to be exercise duration dependent. All initial bouts reduced the soreness associated with the second bout, and no increases in CK were recorded following the second bout. Thus, skeletal muscle adaptation could be induced by a single bout of relatively few eccentric contractions, and increasing prior bout duration did not secure an increased prophylactic effect.

Indirect indices of collagen breakdown (serum type 1 collagen concentration and plasma hydroxyproline) following concentric and eccentric exercise protocols indicated that connective tissue breakdown may accompany eccentric exercise-induced muscle damage. Further analysis of collagen breakdown products in urine (pyridinoline, hydroxyproline, and hydroxylysine) indicated that connective tissue may be injured following unaccustomed eccentric exercise. Although mechanisms initiating collagen breakdown could not be determined, it was suggested that a localised accumulation of collagen breakdown products following eccentric exercise may initiate further connective tissue breakdown via the provocation of inflammatory cell margination into the muscle endomysium and perimysium.

The susceptibility of untrained human skeletal muscle to eccentric exercise-induced damage has been demonstrated. Also, evidence of collagen breakdown following eccentric muscle contractions has been reported. Further work on the mechanisms of muscle connective injury during and after exercise is required.

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Chapter 1.

Introduction.

1.0. Summary.

Experimentally induced damage to skeletal muscle can be used to study the damage and repair processes associated with muscle tissue injury. This review will attempt to summarise the mechanisms of muscle damage and adaptation, examine indirect indices of muscle damage, and consider the role of connective tissue in the post exercise injury reaction.

Damage to skeletal muscle can result from unaccustomed exercise, particularly when the exercise involves predominantly eccentric muscle contractions (for reviews, see: Armstrong, 1984; Armstrong, 1990; Evans and Cannon, 1991; Faulkner et al, 1993; MacIntyre et al, 1995). During an eccentric muscle contraction, tension is developed while the muscle is lengthening, and despite numerous theories, the mechanisms of how this type of muscle contraction causes damage to skeletal muscle are not fully understood. Exercise-induced muscle damage following eccentric muscle contractions has also been called contraction-induced muscle injury (Lapier et al, 1995).

Initial myofibril disruption incurred during eccentric exercise, may predispose some fibres to progressive degeneration (for review, see: Armstrong et al, 1991). Why some fibres appear susceptible to this secondary degenerative cascade is not known at present. Connective tissue structures distribute the forces produced during muscle contraction, and high forces associated with certain types of eccentric contractions may predispose these structures to exercise-induced injury (for review, see: Stauber, 1989). However, the mechanism of injury to connective tissue during eccentric muscle contraction is not well understood. Assessment of exercise-induced muscle damage can be made from muscle biopsy specimens (e.g. Jones et al, 1986; Friden et al, 1983; Newham et al, 1983; Round et al, 1987), or from analysis of indirect markers (e.g. Clarkson et al, 1992; Rodenburg et al, 1993), although both methods have their limitations. Direct evidence of muscle damage obtained using an invasive biopsy procedure may confirm the presence of muscle injury, although this procedure may not be informative about muscle performance. However, repeated indirect measurements taken from a damaged muscle, e.g. isometric force, may also affect muscle repair/regeneration.

Extensive work in the area of muscle damage has been carried out using animal models, e.g. rats (Armstrong et al, 1983; Van der Meulen et al, 1991; Lynn and Morgan, 1994), mice (Zerba et al, 1990; Sacco and Jones, 1992; Warren et al, 1993), and rabbits (Lieber and Friden, 1988; Lieber et al,

1991; Lieber et al, 1994; Hasselman et al, 1995). The assumption that animal studies are comparable with human work cannot be guaranteed. However, since some questions arising from animal work have been further examined by the studies in this thesis, this review cites both animal and human studies.

This literature review is structured in the following way:

1. Mechanical origins of initial damage,
2. The secondary degeneration cascade,
3. Indirect indices as evidence of tissue damage,
4. Exercise-induced connective tissue damage,
5. Adaptation to eccentric exercise,
6. Study aims.

1.1. Mechanical origins of initial damage.

Eccentric exercise-induced muscle damage appears to be initiated by a mechanical stimulus incurred during the exercise period (for review, see: Friden and Lieber, 1992). This damage is visible using electron microscopy (e.g. Friden et al, 1983; Lieber et al, 1991), and has been characterised in a number of species following a variety of exercise protocols. Irregularities in normal muscle architecture have been reported immediately following a variety of damaging exercise protocols, such that normal sarcomere register appeared disorganised after downhill running in rats (Armstrong et al, 1983) and following electrical stimulation of mouse muscle (Brooks et al, 1995). Lieber et al (1991) reported abnormal type 2 fibre morphology in eccentric exercised rabbit skeletal muscle, with disorganisation of the sarcomeric band pattern and extension of the Z disc into adjacent A bands. Jones et al (1984) reported vesiculation of the sarcoplasmic reticulum, swelling and disruption of the mitochondria, and loss of Z disc material in exercised mice skeletal muscle in vitro. In humans, the damage incurred during eccentric muscle contractions presented as focal myofibril disruption and subcellular Z disc streaming (Friden et al, 1983; Newham et al, 1983; Jones et al, 1986). Thus it appears that disruption at the subcellular level is common to eccentric exercise-induced muscle damage in a variety of mamalian skeletal muscle. This damage has been attributed to a variety of mechanical stimuli during performance of the eccentric contractions, although different exercise models have examined the importance of these stimuli in more detail.

1.1.1. Muscle force, stress, and strain.

A mechanical factor which may be important in exercise-induced muscle damage is muscle force (Faulkner et al, 1993). Higher muscle forces are produced by fewer active motor units during an eccentric muscle contraction, and this may predispose the contracting motor units to higher stresses compared to concentric contractions (see Fig.1.1.).

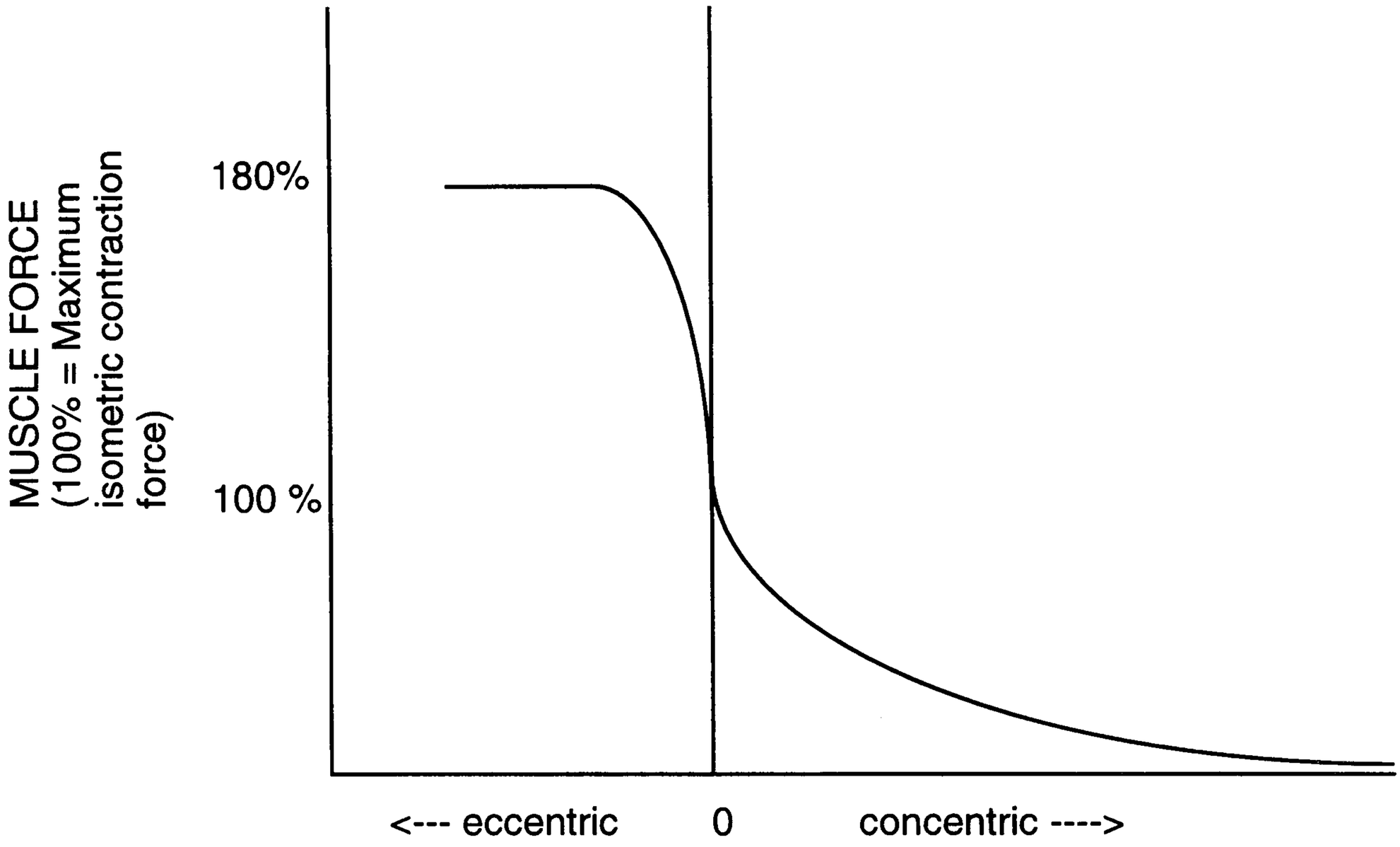


Fig. 1.1. Muscle force-velocity relationship.
(from Friden and Lieber, 1992)

The contribution that contraction force played in muscle damage was originally reported by Hough (1902), where soreness was "much more marked with those muscles which gave the strongest contraction". Using a combination of eccentric and concentric contractions, Tiidus and Ianuzzo (1983) presented evidence which suggested that more damage was caused by high-intensity short-duration exercise, compared to low-intensity long-duration exercise. When muscle strain rate and strain magnitude were controlled (using a work balanced study design), high forces during the initial stages of an eccentric exercise bout appeared to be a major determinant of subsequent muscle damage (Child et al, 1995).

Both stress and strain have been implicated in the initial events of muscle damage. Stress can be defined as the force per unit area acting on a material and tending to change it's dimensions, and strain as the deflection (with respect to original dimension) induced by an applied force. Working on rabbit skeletal muscle, Lieber and Friden (1993) reported that it was not high force that appeared to cause muscle damage during eccentric contractions, but the magnitude of strain during 'active lengthening'. When high forces were obtained by applying a late stretch to a tetanic isometric contraction, no differences in muscle contractile properties were observed when compared with low forces produced by an early stretch protocol (Lieber and Friden, 1993). However, these authors acknowledged that changes in tendon compliance with different muscle forces may affect the strain magnitude within the active muscle, and muscle-joint mechanics needed to be considered when attempting to determine strain magnitudes in vivo.

Brooks et al (1995) used single eccentric contractions of mouse muscle to examine the relationship between both strain and average force on the severity of contraction-induced injury. The decrease in isometric contraction force was used as a quantitative measure of muscle injury, and examination of stretched muscle using electron microscopy revealed disorganisation of myofilaments, misalignment of adjacent sarcomeres, and in some cases, over stretched sarcomeres with widened Z discs. Following single stretches of tetanic muscle, Brooks et al (1995) reported that the work done (average force x strain) to stretch the muscle was the "best predictor of the magnitude of injury". Evidence that muscle damage was more pronounced following eccentric exercise at long muscle length compared to short muscle length has been reported (Jones et al, 1989; Child et al, 1995). However, it may be that the strain ranges used in experimental models and during in vitro work are not experienced in humans in vivo.

The force generated by stretching a "passive muscle" (i.e. no active motor units) is dependent on the inherent resistance to strain in parallel elastic structures such as intramuscular cytoskeletal proteins (e.g. titin), basal lamina, and parallel connective tissue. For passive mouse muscle (Brooks et al, 1995), single stretches of 50 % strain were necessary to produce a significant deficit in maximum isometric contraction force. These authors reported force deficits of 93 % following single strains of 60 % magnitude in passive muscle, although the partial recovery of the force loss during the following 30 min suggested that muscles were not stretched beyond their range of extendibility. However, work by McCully and Faulkner (1986) and Faulkner et al (1989) reported that passive stretches did not

result in skeletal muscle damage in mice, a finding similarly reported by Jones et al (1989) using human muscle. Thus, it appears that the effect of lengthening passive muscle (and the rate of lengthening) appears to be important in certain types of muscle injury e.g. acute muscle rupture, and further work on human skeletal muscle is needed.

1.1.2. Sarcomere heterogeneity.

Certain aspects of sarcomere physiology, namely the force-velocity curve (see Fig. 1.1.), and the length-tension relationship (see Fig. 1.2.), may contribute to the mechanical causes of contraction-induced injury. It has been suggested that eccentric exercise-induced muscle damage may be attributed to imbalances between adjacent sarcomere lengthening velocities (Friden and Lieber, 1992), and/or non-uniformities in sarcomere length throughout the active fibres (Morgan, 1990).

The muscle force velocity curve describes a disproportionate increase in force at certain lengthening velocities, a condition which is inconsistent with all shortening velocities. Friden and Lieber (1992) suggested that adjacent sarcomeres may not have an identical force velocity relationship. Therefore, at certain lengthening velocities there is a potential for an imbalance in adjacent sarcomere tension which may lead to shear forces between sarcomeres in an active fibre. During eccentric muscle actions, inter-sarcomere shear forces may provide an explanation for the Z disc disruption observed immediately post exercise.

Tension generated by a sarcomere is dependent on the extent of actin-myosin overlap, and therefore the length of the sarcomere is critical in tension development (see Fig. 1.2.). It has been postulated (Morgan, 1990) that variations in myofilament overlap along 'in series' sarcomeres, predispose those sarcomeres of longer lengths (on the descending limb of their length tension curve) to contraction-induced injury. Using a mathematical model, Morgan (1990) predicted that due to random variations in the lengths of series sarcomeres, stretches take place non-uniformly by the rapid and uncontrolled lengthening of sarcomeres beyond myofilament overlap. This condition was predicted by Morgan (1990) to occur in relatively few sarcomeres. Morgan (1990) considered that sarcomeres with a tension generating capacity that

decreased with increasing length would be prone to instabilities, and

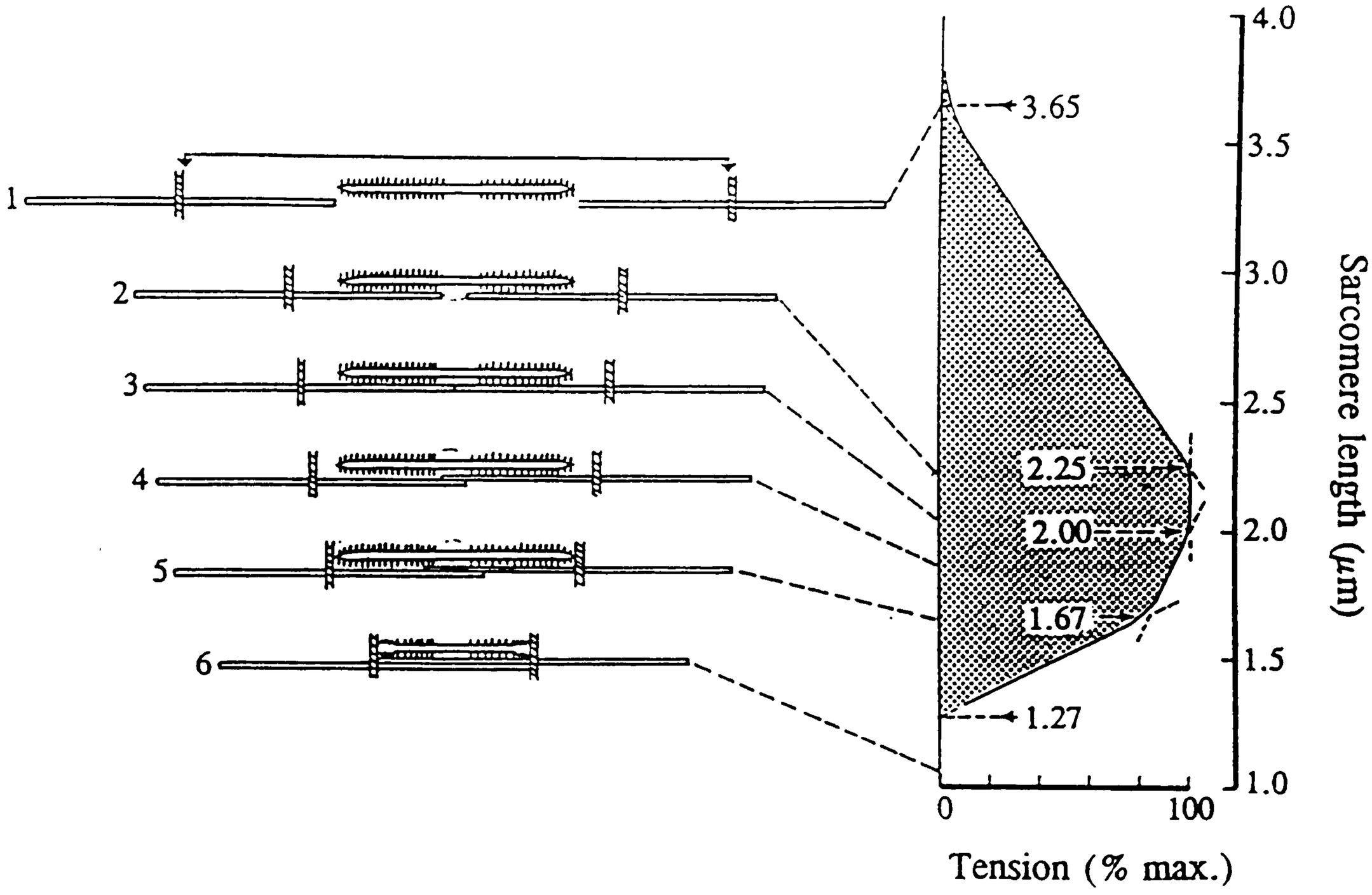


Fig.1.2. The sarcomere length-tension relationship (from McComas, 1996). Positions 1 to 6 indicate progressively increasing myofilament overlap corresponding to a progressive decrease in sarcomere length.

this may result in 'over-stretched' or 'popped' sarcomeres. Myofilament overlap, and therefore tension, in sarcomeres on the descending limb of their length tension relationship could be further reduced by higher tensions produced by series sarcomeres on the plateau or ascending limb of their length tension relationship. Such a random distribution of sarcomere lengths may partly explain the focal nature of contraction-induced injury, although sarcomeres with the shortest lengths are usually located a the ends of fibres. Experimentation on permeabilised fibre segments (Macpherson et al, 1997) demonstrated that the range of sarcomere lengths increased during a maximum isometric tetanus, and that regions which contained the longest sarcomere lengths (those on the descending limb of their length tension relationship) contained the majority of damaged sarcomeres following a single stretch of 40 % strain relative to optimum length.

Any mechanical overload that disrupts muscle homeostasis may lead to the damage observed in skeletal muscle following exercise. Whether muscle membranes, calcium handling mechanisms, structural cytoskeletal proteins, or connective tissue proteins are sites of initial exercise-induced mechanical damage, it seems that eccentric contractions in untrained muscle predispose some fibres to damage. A number of mechanical factors appear to be involved in the initial damage processes, and the relative contribution of each factor has received some attention by previous authors (see above). In human muscle in vivo, it seems likely that all factors work collectively to contribute in some way to the development of injury following unaccustomed eccentric exercise.

1.2. The secondary degeneration cascade.

An initial mechanical event may predispose some fibres to secondary degeneration processes, and this has been demonstrated in muscle biopsy specimens examined histologically taken some time after the exercise bout (e.g. Jones et al, 1986). Histological evidence of muscle fibre degeneration was observed in mouse muscle following lengthening contractions (McCully and Faulkner, 1985) although not following isometric or concentric exercise protocols. This evidence of damage reached a peak at 3 days post exercise, with infiltration of the muscle tissue by macrophages, and progressive degeneration being observed two to four days following lengthening contractions. In humans, two days post eccentric exercise, Friden et al (1981) reported sub-cellular level abnormalities with frequent focal disturbances of the characteristic cross-striated band pattern. At the ultra-structural level, disturbances were found to originate from the Z disc (Friden et al, 1981). The author's interpretation of these findings was that high myofibrillar tension during the eccentric muscle actions resulted in some mechanical disruption of the Z disc, although structural disturbances may also be secondary, possibly resulting from an activation of lysosomal enzymes with concomitant inflammation. Also, in human skeletal muscle, delayed cellular infiltration was observed between 9 and 14 days following eccentric exercise (Jones et al, 1986). These authors also demonstrated a delayed time course of increases in serum creatine kinase activity (4 to 6 days post exercise), a time course paralleled by an increased uptake of technetium pyrophosphate into the exercised muscle. However, Jones et al (1986) noted that cellular infiltration was seen as a response to damage

rather than the cause, and the reason for the delay between the exercise bout and the appearance of damage remained unclear.

1.2.1. Calcium activated processes.

A theoretical link between initial mechanical damage and the onset of secondary degeneration processes is a loss of intracellular calcium ion homeostasis. Duncan (1978) suggested a role for increased myoplasmic $[Ca^{2+}]$ in the aetiology of myofibril degeneration, and a rise in intracellular $[Ca^{2+}]$ has been implicated in a variety of myopathies (e.g. Duchenne Muscular Dystrophy). Similarities between eccentric exercise-induced damage to skeletal muscle and dystrophic muscle possibly suggested that impaired calcium handling is common in both scenarios (Stauber et al, 1991). Although a transient increase in intracellular $[Ca^{2+}]$ is essential for muscle contraction, prolonged elevation may be a precursor of myofibril degeneration. Rapid, uncontrolled Ca^{2+} entry into the cell may result from small changes in calcium permeability, due to the large concentration gradient. Exercise-induced disruption of the sarcolemma, whether mechanical or metabolic in origin, could allow Ca^{2+} to enter the myoplasm down an electrochemical gradient. Also, an impaired ability of the SR to sequester Ca^{2+} from the cytosol (for review, see: Byrd, 1991) may contribute to the prolonged elevation in intracellular $[Ca^{2+}]$.

Secondary degeneration in mouse skeletal muscle preparations (Jones et al, 1984) has been attributed to raised intracellular $[Ca^{2+}]$, and the damage was attenuated by withdrawing external calcium from the surrounding medium. Jones et al (1984) suggested that the uncontrolled entry of Ca^{2+} into skeletal muscle initiated more extensive damage, thus leading to the release of intramuscular constituents (e.g. lactate dehydrogenase) some 60-90 minutes after the end of muscle stimulation. Armstrong's (1984) theoretical model of exercise-induced muscle damage cited membrane damage and disturbed Ca^{2+} homeostasis as causative mechanisms in the aetiology of muscle fibre necrosis and secondary degeneration. Armstrong (1984) suggested that this was attributed to increased activation of calcium sensitive cytosolic phospholipases and proteases. In a later review, Armstrong et al (1991) proposed a model of exercise-induced muscle damage based on the principle of calcium overload and calcium activated degenerative processes (termed the autogenetic phase). Although a role for increased protease activity could not be demonstrated in either mice muscle stimulated in vitro (Jackson et al,

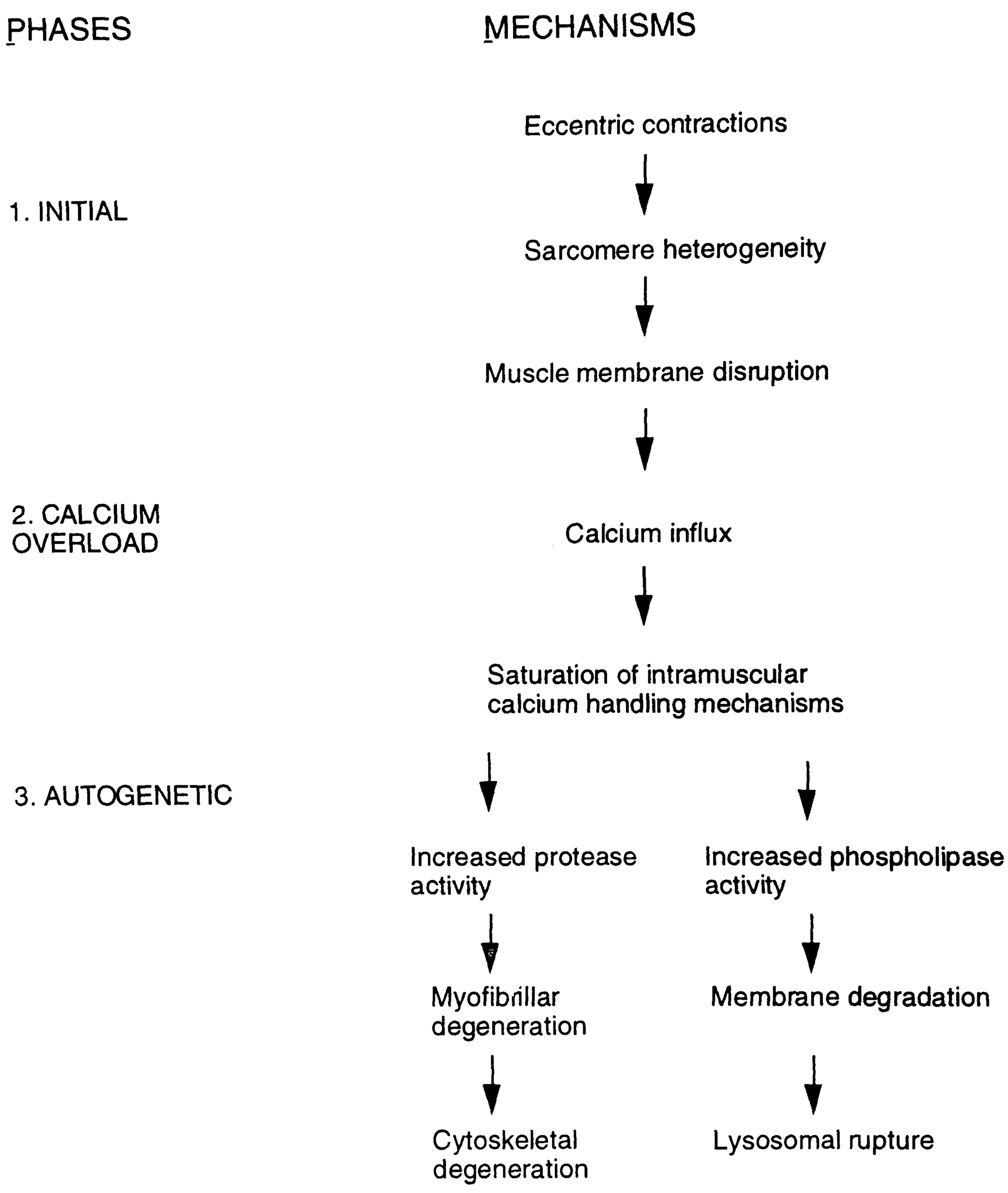


Fig. 1.3. Proposed model of the phases of exercise-induced muscle damage. (From Armstrong et al, 1991).

1984), or in chemically skinned amphibian skeletal muscle (Duncan, 1987), the use of phospholipase A_2 inhibitors appeared to reduce enzyme efflux from stimulated skeletal muscle (Jackson et al, 1984; Duncan and Jackson, 1987). Activation of intramuscular phopholipase by intracellular Ca^{2+} may cause a degeneration of muscle membranes, thus giving rise to the characteristic leakage of enzymes from damaged muscle. Phospholipase A_2 will act on membrane phospholipids, giving rise to free fatty acids, which in turn may act as detergents causing further membrane damage (Jackson et al, 1984).

1.2.2. Muscle protease activation.

Activation of both lysosomal and non-lysosomal proteases have been suggested as possible mechanisms of secondary fibre degeneration (Armstrong, 1991), although evidence of increased Ca^{2+} activated protease activity in exercised human muscle is limited. Belcastro (1993) reported an increase in rat muscle protease (non-lysosomal Ca^{2+} activated neutral protease) activity following exercise, and this was attributed to an increased sensitivity to Ca^{2+} and an enhanced susceptibility of myofibril substrate proteins (e.g. desmin, vimentin, and alpha-actinin). The mechanism of protein substrate modification was not identified, although Belcastro (1993) speculated that covalent modification of proteins (e.g. altered oxidation-reduction status) may be a targeting mechanism for selected protein degradation. Using mice skeletal muscle, Vihko et al (1978) reported a delayed increase (2 days post exercise) in lysosomal acid hydrolase activity following exhaustive treadmill running. Increased activity of lysosomal enzymes have been found to be associated with degenerative processes in skeletal muscle following a period of ischaemia (Shannon et al, 1974; Shannon and Courtice, 1975), although elevated beta-glucuronidase activity was also demonstrated during muscle regeneration. Increased beta-glucuronidase activity was reported in untrained mice muscle subjected to exhaustive training (Vihko et al, 1979), and increased beta-glucuronidase activity was also reported in human skeletal muscle following unaccustomed eccentric exercise (Child et al, 1996). However, the presence of macrophages in exercise-induced damaged tissue may contribute to the overall measurement of acid hydrolase activity. Thus, conclusive evidence

for the involvement of proteases and lysosomal enzymes in exercise induced-muscle damage is unavailable at present.

1.2.3. Inflammation.

A theoretical link between inital damage and secondary muscle degeneration has been proposed which cites inflammation as a major cause of secondary degeneration (for reviews, see Smith, 1992; MacIntyre et al, 1995). Exercise-induced muscle damage has been proposed as an experimental model to study muscle inflammatory diseases (Round et al, 1987), although at present the results of studies examining inflammation following eccentric exercise are equivocal (Nosaka and Clarkson, 1996). It has been suggested that the delayed nature of muscle soreness following unaccustomed eccentric exercise may indicate some inflammatory process initiated by damage to muscle connective tissue (Jones and Round, 1990). Initial muscle injury may lead to a localised accumulation of cytotoxic damage products which may be a precursor for inflammatory cell margination in the muscle. Connective tissue breakdown products may play a chemo-attractant role in this margination (Stauber, 1989), although exact mechanisms are not known at present. An exercise-induced inflammatory response may cause further tissue damage (including proteolysis by infiltrating neutrophils and macrophages), therefore prevention of inflammation may attenuate some aspects of muscle damage. The use of non-steroidal anti inflammatory drugs in the treatment of exercise-induced muscle damage has been studied in animals (Mishra et al, 1995) and humans (Kuipers et al, 1985; Donnelly et al, 1988; Hasson et al, 1993), although overall results are equivocal. It is probable that inflammatory processes are a part of certain experimental exercise-induced muscle damage models, although the time course of tissue swelling and soreness (classical symptoms of inflammation), and cellular infiltration do not coincide. Also the variable response of anti-inflammatory medications used to alleviate muscle soreness adds evidence to suggest atypical inflammation following exercise-induced muscle damage. Further work on determining the role of inflammation in exercise-induced muscle damage is required.

Although the classification of experimental exercise-induced muscle damage into initial and secondary processes may be artificial, the delayed

degeneration of some skeletal muscle fibres following exercise remains an interesting phenomena. Calcium activated autolytic processes, possibly triggered by prolonged elevation of intracellular [Ca^{2+}], may be involved in muscle fibre degeneration, or an exercise-induced inflammatory process may initiate further muscle tissue breakdown. However, it is probable that in human skeletal muscle damaged by unaccustomed eccentric exercise, a combination of processes cause the delayed damage. Further work is required to examine the mechanisms of secondary degeneration of muscle fibres, and on intervention strategies which may limit the degeneration cascade.

1.3. Indirect indices as evidence of tissue damage.

The heterogeneous distribution of damage in exercise-induced damaged muscle increases the risk of sampling errors associated with a needle biopsy, and frequency of sampling may also be a problem with this technique. The choice of sampling site may also be a problem if attempts are made to examine connective tissue disruption (e.g. myotendinous junction). Although demonstration of morphological damage in muscle fibres may be needed to confirm muscle injury, the widely dispersed nature of the damage makes quantitative assessment difficult. Indirect markers of muscle damage have been developed and used as evidence of tissue injury (e.g. Clarkson et al, 1992; Rodenburg et al, 1993). Indirect markers have the advantage of being generally less invasive than direct muscle sampling, can be repeated over many days, and can be informative about the muscle performance as a contracting unit, ie. from excitation through to limb movement.

1.3.1. Muscle soreness.

Delayed onset muscle soreness (DOMS) is commonly used in studies of exercise-induced muscle damage. Although the mechanisms which produce the sensation of soreness in eccentric exercised muscle are not fully understood, the measurement of soreness can be a useful indicator of the possible presence of tissue damage. Whether subjective ratings of perceived soreness are measured using analogue scales from 1 to 10 (eg. 1= no soreness to 10= very, very sore), or using a pressure myometer technique (Eston et al, 1996; Teague and Schwane, 1996), the

quantification of DOMS may be a useful tool in the assessment of muscle injury. Early evidence of the apparent association between muscle soreness and muscle damage was reported by Hough (1902), and the predominance of this soreness following eccentric muscle contractions was reported by Asmussen (1956). It has been suggested (Jones and Round, 1990) that exercise-induced pain was due to an inflammation of muscle connective tissue, such that sensory afferent neurones primarily located in connective tissue were activated by inflammatory mediators and/or connective tissue breakdown products. Evidence of collagen breakdown and muscle soreness following unaccustomed eccentric exercise in humans has been reported (Abraham, 1977) and this may suggest a relationship between DOMS and connective tissue disruption, however the exact mechanisms of DOMS are not fully determined.

1.3.2. Contractile force.

Because of limitations involved in the direct assessment of muscle damage and difficulties involved in quantitative evaluation of morphological damage, the deficit in the development of maximum isometric contraction force (MVC) may be a valid measure of the totality of the damage (Brooks et al, 1995; Faulkner et al, 1993). Loss of contractile force at different angular velocities of limb movement following eccentric exercise has been reported (Friden et al, 1983; Eston et al, 1996), and these methods of assessment may be useful in identifying damage within a certain fibre population. Force loss immediately post exercise may reflect the combined effects of muscle fatigue and initial exercise-induced damage. Clarkson et al (1992) reported a decline in human forearm flexor MVC of more than 50 % following high force eccentric exercise, and a deficit in strength was still evident up to 10 days post exercise. Although maximum force loss may occur immediately post exercise, a gradual recovery in MVC during the days after eccentric exercise has previously been reported (Clarkson et al, 1992; Nosaka et al, 1991). However, it has been demonstrated that during this recovery period evidence of progressive ultra-structural damage may develop (Friden et al, 1983; Jones et al, 1986). A biphasic force loss has been reported following eccentric exercise, (MacIntyre et al, 1996), and these authors explained this in terms of initial fatigue and partial recovery, followed by secondary degeneration. Following electrically stimulated eccentric muscle contractions, maximum loss of MVC has been reported 3 days post exercise in mice muscle (22 % of pre-exercise MVC) by McCully and Faulkner (1985),

and 2 days post exercise in rabbit skeletal muscle (Lieber et al, 1994). This continued decline in MVC during the days post exercise may reflect the increasing ultra structural damage observed by previous authors. At present it is unclear as to why contractile force may fully recover despite extensive cellular infiltration within the muscle many days after the exercise.

1.3.3. Force-frequency characteristics.

Changes in the force-frequency relationship of skeletal muscle have been used as evidence of exercise induced muscle damage (e.g. Newham et al, 1983). Low-frequency fatigue, or long lasting fatigue (Edwards et al, 1977) has been characterised as a chronic force loss at low compared to high frequencies of stimulation. A feature of low-frequency fatigue is that it may persist for hours or days in the absence of gross metabolic or electrical disturbance to the muscle (Jones, 1996). It has been suggested (Jones, 1981; Westerblad et al, 1993) that the disproportionate loss of force at low frequencies of stimulation following eccentric exercise may be due to impaired Ca^{2+} release from the sarcoplasmic reticulum (SR).

Due to the sigmoidal relationship between force production and intramuscular calcium ion concentration (Jones, 1996), tetanic contractions produced by low frequencies of stimulation are more sensitive to fluctuations in the quanta of calcium released from the SR. An impairment of calcium release from the SR will therefore disproportionately affect the forces produced at low stimulation frquencies (when small changes in intracellular Ca^{2+} have a large effect on tetanic force), compared to higher stimulation frequencies (when tetanic force is insensitive to changes in intracellular Ca^{2+} concentration). Using mouse skeletal muscle, McCully and Faulkner (1985) demonstrated that lengthening contractions resulted in a significant force loss at low stimulation frequencies and this indicated the presence of muscle injury. Similar findings in human muscle (Newham et al, 1983; Saxton and Donnelly, 1994) have also been used as evidence of exercise induced muscle damage. A muscle length dependent component of the force frequency relationship has been suggested (Sacco et al, 1994; Jones, 1996), and therefore the low-frequency fatigue observed following eccentric exercise may be indicative of changes in muscle length, an hypothesis consistent with the findings of Lynn and Morgan (1994). The reasons for the differences in the time course of recovery of low frequency fatigue and recovery of isometric force following some eccentric exercise protocols are as yet undetermined.

1.3.4. Circulating enzyme activities.

Numerous studies have examined the release of intramuscular constituents into the circulation following muscle damage, and an increase in the activities of circulating muscle specific proteins may be suggestive of damage to the plasma membrane of muscle fibres. For example, systemic activities of creatine kinase (Schwane et al, 1983), lactate dehydrogenase (Nosaka et al, 1992) , and concentrations of myoglobin (Nosaka et al, 1992; Rodenburg et al, 1993), and myosin heavy chain fragments (Mair et al, 1992) have all been used as indirect indices of muscle damage. However, variability within subjects and problems associated with clearance kinetics should be considered when examining changes in serum/plasma enzyme activities following eccentric exercise. Van der Meulen et al (1991) studied the release of enzymes into the circulation from rats following an uphill run of either 1.5 h or 2.5 h, and attempted to estimate the amount of tissue damage from blood enzyme parameters. These authors presented evidence which suggested that the amount of structural damage observed histologically was significantly lower than the amount that could be estimated by plasma enzyme activities. Similar findings were later reported (Volfinger et al, 1994) using equine muscle, where "the amount of damaged muscle may be negligible following a 60 km exercise test" despite elevated plasma CK activity, and that only circulatory CK activity levels >10,000 $IU.l^{-1}$ could be used to demonstrate myolysis. Kolumainen et al (1995) demonstrated that serum CK activity may increase without concomitant muscle damage, and that muscle damage may occur without significant increases in serum CK activity. A possible criticism of these conclusions is that although damage may not be visible under light microscopy, this does not preclude structural damage to the muscle membrane which may allow for enzyme efflux.

A variety of values for serum CK activity in humans following different exercise protocols have been reported. Downhill running protocols typically result in a CK activity of 250 - 500 $IU.l^{-1}$ some 12 - 48 hours post exercise (Byrnes et al, 1986), whereas high force eccentric contractions may result in values of 80,000 $IU.l^{-1}$ some 6 days post exercise (Jones et al, 1986). Eccentric exercise of a smaller muscle mass i.e. the biceps brachii, typically resulted in CK values of about 2,500 $IU.l^{-1}$ 4-5 days post exercise (Clarkson et al, 1992), although these authors noted that values for "high responders" could exceed 6000 $IU.l^{-1}$ some 4 days post exercise. These higher values i.e. >6,000 $IU.l^{-1}$, were also reported by Donnelly et al (1992) using an exercise

model consisting of eccentric contractions of both the forearm flexors and extensors. Thus, it appears that depending on the muscle group used and the exercise protocol, the increased activities of CK in the blood may be used as an indicator of skeletal muscle damage. However, it should be emphasized that increased circulating CK activity is only an indirect indication of possible skeletal muscle damage, and can not be used as a quantitative measure of the extent of muscle damage.

Indirect indices of muscle damage may not confirm the presence of muscle damage, although they have a role in monitoring the effects of exercise and the time course of recovery from injury. There is a need for the development of more specific markers of muscle damage, and a quantitative relationship between actual muscle fibre injury and an indirect marker needs to be established. When a battery of indices of exercise-induced muscle damage are used to study the damage and repair processes, an interesting time course of events may be established. The mechanisms underlying the timing of the changes in indirect indices observed after eccentric exercise remain undetermined.

1.4. Exercise-induced connective tissue damage.

A network of connective tissue structures distribute the forces produced during muscle contraction and also serves as a supportive structure during normal muscle growth and regeneration from trauma (Lehto et al, 1985; Barlow and Willoughby, 1992). Connective tissue contains both a cellular component and an extracellular matrix composed of a ground substance and fibres. The ground substance comprises of interstitial fluid, glycoproteins, and glycosaminoglycans. Fibres found in the matrix are typically collagen, elastin, and reticullar fibres, of which collagen is the most abundant. The collagen sub-types identified immunohistochemically in the connective tissue of skeletal muscle are type 1 in the epimysium and perimysium, type 3 in the endomysium, and types 4 and 5 in the basal lamina of the fibres (Duance et al, 1977). Epimysium is an extension of the deep fascia surrounding skeletal muscle, and invaginations of the epimysium (perimysium) divide parallel muscle fibres into fascicles. The endomysium (invaginations of the perimysium) penetrates into the interior of each fascicle to separate individual muscle fibres. These structures are continuous with the dense regular connective tissue of the tendon at both

origin and insertion. Muscles and tendon functionally act as a single unit in which muscle is attatched to bone by the tendon. Tendons consist predominantly of type 1 collagen, with approximately 5 % of types 3 and 5. The high forces associated with certain types of eccentric exercise may predispose the connective tissue to exercise-induced injury (for review, see: Stauber, 1989).

1.4.1. Indices of collagen metabolism.

Collagen is a major protein constituent of connective tissue (Prockop et al, 1979) and the levels of organisation of fibrillar collagen is described in Fig. 1.4. Measurement of biochemical markers of collagen synthesis and breakdown may be used to identify the effect of eccentric exercise on connective tissue.

Hydroxyproline and hydroxylysine are imino acids characteristic of, but not exclusive to, collagen. Abraham's (1977) work on exercise-induced muscle soreness and urinary hydroxyproline (HP) excretion in humans used predominantly a 'step test' exercise regimen, although two subjects (from a total of 7) used additional methods, e.g. weight lowering, to induce soreness. Although this author reported a significant correlation between the day of peak soreness and HP excretion, subject's reported a variable time course for these measures, and soreness was reported in both the positive and negative working legs. Abraham (1977) reported no significant differences in the HP: creatinine ratio between the control day and either of the post exercise days, although total HP excreted throughout the experiment was significantly greater than the corresponding control value. The variability in the control and pre-exercise urine HP values were larger than the significant difference between the control and experimental values at 48 hours post exercise. Consequently, the conclusions reported by Abraham (1977), where "...soreness inducing exercise is related to an increased hydroxyproline excretion", and "...delayed muscle soreness observed 24-48 hours post exercise is mostly correlated to alterations in the muscle connective tissue ", may not be justified by the data presented.

Elevated levels of plasma hydroxyproline have been reported in subjects with connective tissue injuries (Murguria et al, 1988), although despite a significant increase in serum CK activity following a downhill running protocol, Wheat et al (1989) reported no increases in the urine concentration of hydroxylysine. Although sensitive and specific assays exist for hydroxyproline, it's usefulness as a marker of collagen degradation is

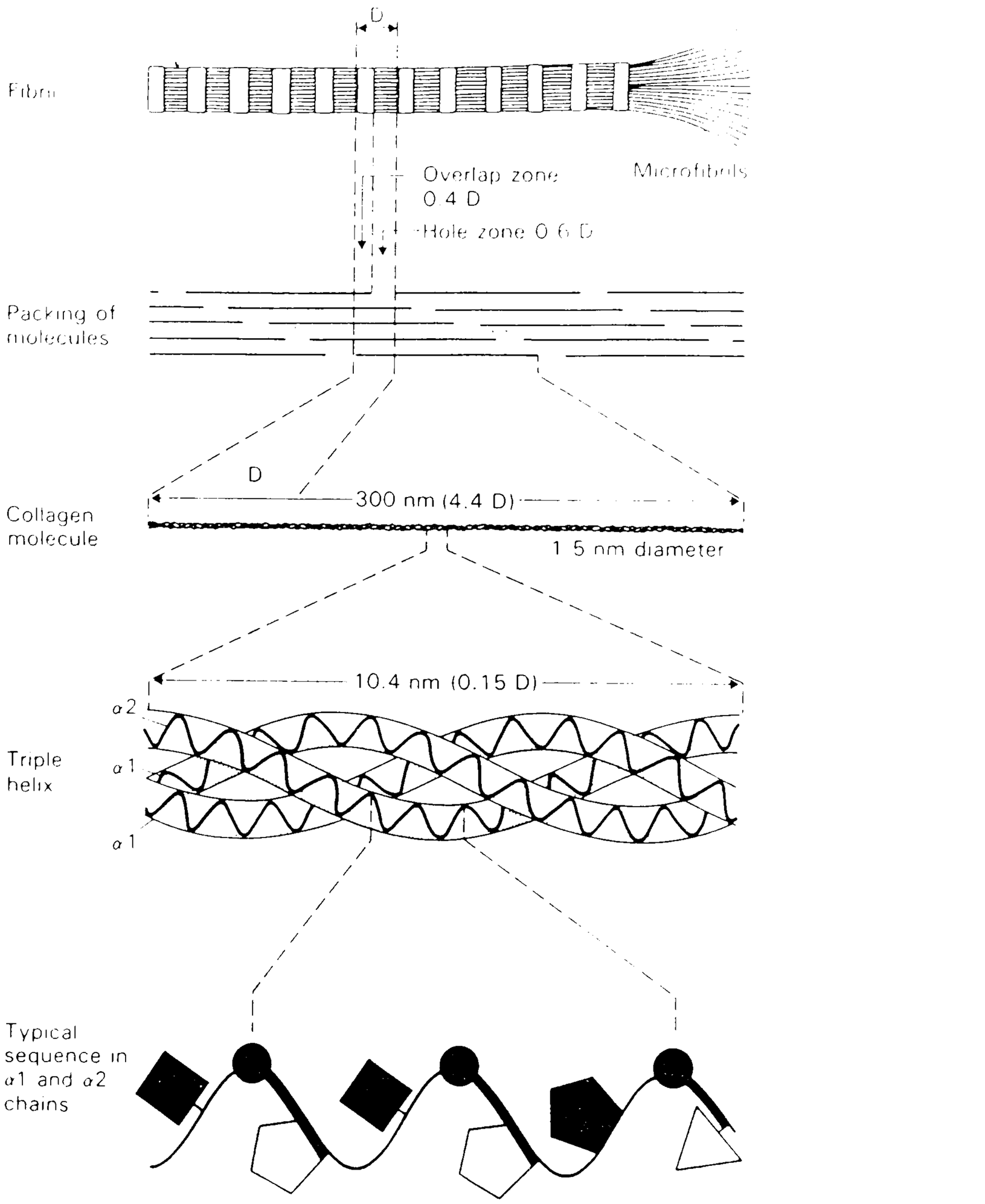
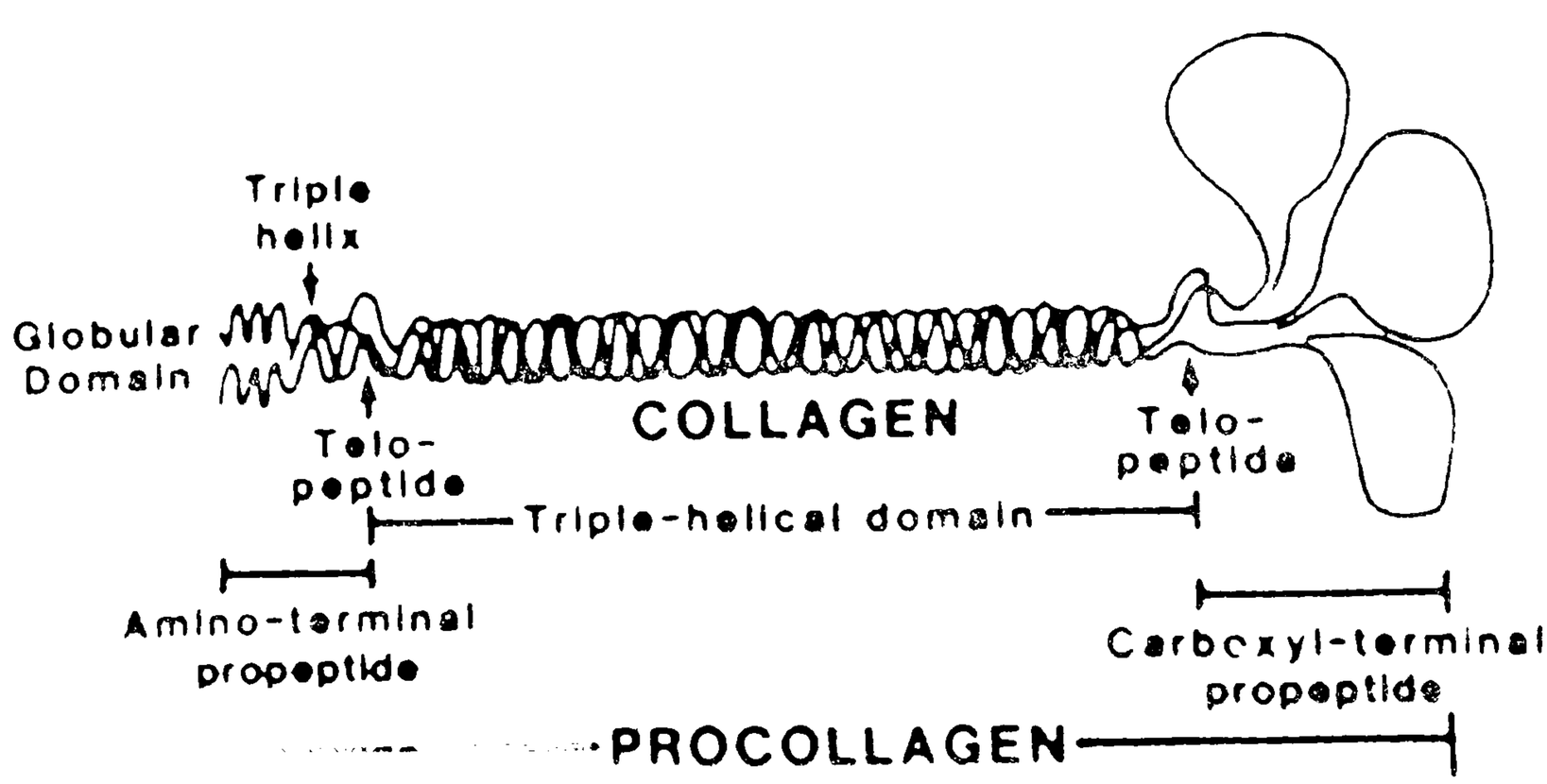


Fig. 1.5. Levels of organisation of the fibrillar collagens (from Komi, 1992).

Fig. 1.6. (Below) Procollagen, the pre-cursor of collagen.



limited. It is not specific with respect to collagen type, and less than half of excreted hydroxyproline is derived from bone turnover, the remainder being from collagen propeptides, complement, and intracellular catabolism of over-produced newly synthesised collagen (James et al, 1996). Other markers of collagen metabolism have shown a variable response to different exercise protocols. Takala et al (1986) reported an increased serum concentration of the amino propeptide of type 3 procollagen (cleaved during the final stages of collagen synthesis, see Fig.1.5.) following a 24 hour run, but not after 24 hours of cross-country skiing (Takala et al, 1989). This marker of new collagen synthesis also showed an increased serum concentration following high intensity concentric exercise (Virtanen et al, 1993). Kristofferson et al (1995) used serum biochemical markers of bone formation and degradation, carboxyterminal propeptide of type 1 procollagen and the carboxyterminal telopeptide region of type 1 collagen respectively, to investigate the effects of short term high intensity exercise on bone remodelling. These authors recorded no significant changes in these markers at either 5 mins. post exercise or 1 hour post exercise, although it was acknowledged that the short time span may not be suitable for studies of acute exercise bouts and collagen metabolism.

Using rat skeletal muscle, Lapier et al (1995) examined the effect of immobilisation at different muscle lengths on subsequent susceptibility to contraction-induced muscle injury. By comparing the ratio of total connective tissue area to muscle fibre area, these authors demonstrated that muscles immobilised in a lengthened position had a greater concentration of connective tissue than muscles immobilised in a shortened position. Lapier et al (1995) reported that the muscle's increase in intramuscular connective tissue and the accompanying resistance to contraction-induced injury were consistent with the hypothesis that increased connective tissue contributed to the reduced susceptibility to injury. However, these authors acknowledged that changes in the connective tissue architecture (e.g. increased intermolecular cross-links in muscle fixed in a lengthened position), and not just proliferation of endomysium and perimysium, could account for the resistance to contraction-induced injury. Chronic eccentric training has been shown to induce changes in rat muscle connective tissue (Stauber et al, 1996), such that following a 4 week period of forced lengthening contractions (50 repetitions every other day), an expanded extracelluar matrix with increased amounts of fibronectin and concanavalin A were observed. Changes in intramuscular connective tissue appeared to be contraction velocity dependent, with training using fast velocity eccentric

muscle actions causing a greater increase in connective tissue proliferation than slow velocity contractions (Stauber et al, 1994; Stauber et al, 1996).

Collagen biosynthesis is characterised by a large number of post-translational modifications of the polypetide chains, and these modifications affect the quality and stability of the collagen molecule. A 52 % increase in the activity of prolyl 4-hydroxylase was recorded in the rat soleus muscle 2 days after a single bout of exhaustive running, although this did not produce significant increases in the concentration of muscle hydroxyproline (Karpakka et al, 1990). It may be possible that despite an increased synthesis and post translational modification of new collagen, this does not match the increased collagen catabolism. The effect of single or repeated bouts of eccentric exercise on the activities of collagen post translational modification enzymes requires future study as only limited research in this area has been carried out.

1.4.2. Collagen cross-links.

Cross-linking of collagen molecules occurs in the extracellular compartment and involves the oxidation of lysine and hydroxylysine residues by lysyl oxidase (for review, see Robins, 1982). The formation of non-reducible cross-links in mature collagen (eg. pyridinoline) confers stability to collagen fibrils, and cross-links released during collagen remodelling are excreted in the urine. There have been recent developments in cross-link assays (for review, see James et al, 1996), and the concentrations of pyridinoline and deoxypyridinoline in urine and serum have been used to monitor the treatment of connective tissue diseases (Uebelhart et al, 1990; Robins et al, 1991). However, the effect of exercise on this marker has not been extensively studied. Connective tissue tensile strength may alter following exercise-induced injury, possibly by removal or formation of collagen cross-links. Medoff (1987) suggested that connective tissue remodelling involved reorientation of new collagen fibres and formation of cross-links, however, it has been suggested that new collagen synthesised in response to exercise lacked mature cross-linking (Curwin et al, 1988) and therefore connective tissue remodelling may involve transient periods of mechanical weakness (Zamora and Marini, 1988).

The extent to which connective tissue structures are affected by unaccustomed eccentric exercise is not clear. Evidence of an increase in

both collagen breakdown and collagen synthesis have been reported, although complications arise when comparing between studies that have used different exercise modalities. Considering the importance of connective tissue in force distribution and the high incidence of connective tissue injury in certain athletic populations (James and Jones, 1990), further work in the area of connective tissue damage following exercise is required.

1.5. Adaptation to eccentric exercise.

Training appears to induce an adaptive change in muscle which can potentially ameliorate the damage from subsequent eccentric exercise. However, the mechanisms of muscle adaptation to eccentric exercise are not fully determined. Early evidence of a training adaptation which diminished an exercise-induced increase in serum enzyme activity was reported by Nuttal and Jones (1968). These authors speculated that the change could be due to "maintenance of the integrity of the muscle cell membrane" following exercise. This was echoed by Clarkson and Tremblay (1988), who speculated that eccentric exercise-induced damage provided a stimulus for "strengthening the membrane and/or surrounding connective tissues against further insult". Ebbeling and Clarkson (1990) suggested that adaptation was not at the site of the contractile elements, and speculated that the point where CK ceased to be released from damaged tissue indicated the onset of repair and "strengthening" of the membrane. Since the efflux of muscle proteins into the circulation can be reduced with prior eccentric exercise, an adaptation of the membrane such that no changes in permeability occur, is a possible adaptation mechanism.

Jones et al (1986) speculated that preferential damage to type 2 fibres (also shown in biopsy specimens by Friden et al, 1983) may be due to the selective recruitment of this fibre type during eccentric contractions. Thus adaptation could be such that during a repeated bout of eccentric exercise, an altered pattern of fibre recruitment may protect vulnerable or susceptible fibres from damage. An early attempt to explain reduced soreness with training was based on a similar principle (Hough, 1902), such that "trained muscle" was better able to co-ordinate motor unit recruitment, thereby preventing imbalances between contracting and relaxing motor units. If part of the adaptation is a learning effect based on motor control, the stimulus required to induce the effect appeared to be minimal (Nosaka et al, 1991). Neuromuscular adaptation to eccentric exercise, and the possibility of subsequent protection from damage, requires further work.

1.5.1. Susceptible-fibre theory.

Armstrong et al (1983) suggested that an initial unaccustomed exercise bout would damage a pool of stress-susceptible fibres which could result in a large increase in serum CK activity. Subsequent fibre regeneration would result in fewer stress-susceptible fibres and a lower serum CK activity following repeated bouts of exercise. The concept of a small population of susceptible fibres was not supported by McCully and Faulkner (1986), where over 30% of fibres examined histologically revealed overt injury. These authors speculated that exercise-induced injury was not necessarily limited to a small population of susceptible fibres but can involve a large proportion of the fibres in a muscle. Human skeletal muscle biopsy material following a repeated bout of eccentric exercise has not been examined, and therefore the absence of damage following a repeated bout of exercise has not been confirmed. Although speculative, this biopsy material may display distension of the Z discs in fibres affected by the initial mechanical overload (contributing to the force loss seen after repeated bouts), but no evidence of delayed damage or cellular infiltration (possibly resulting in no enzyme efflux). Further work on human skeletal muscle adaptation to eccentric exercise, particularly thorough examination of tissue, is required.

1.5.2. Training specificity and intensity.

The specificity of training required to elicit adaptation was discussed by Armstrong (1984) in terms of both the muscles involved and the type of contractions performed. It was suggested that eccentric muscle contractions were required to induce protection but only in those muscles active during the contractions. Damage to rat skeletal muscle following downhill running was prevented by running protocols which incorporated eccentric contractions, but not by previous episodes of uphill running (Schwane and Armstrong, 1983). Thus, it appeared that an eccentric component in a prior exercise bout was necessary for the adaptation.

Sacco and Jones (1992) speculated that the intensity of the initial training exercise may relate to the degree of subsequent protection afforded to the muscle. Also, Nosaka et al (1991) suggested that the length of the repeated bout effect was related to the "intensity of muscle damage", such that the greater the overload the greater the resulting adaptation. Following

repeated bouts of downhill running in humans (Byrnes et al, 1985), muscle soreness and serum CK activity were reduced when the bout was repeated 3 or 6 weeks later. A single episode of eccentric muscle contractions appeared to protect the muscle from some aspects of damage (Newham et al, 1987; Clarkson and Tremblay, 1988; Sacco and Jones, 1992), although damage may not necessarily be a prerequisite for adaptation (Balnave and Thompson, 1993). Training may cause the muscle to become less susceptible to the initial damage incurred during the exercise. However, the training does not appear to alter the susceptibility of eccentric exercised muscle to acute force loss and changes in force frequency characteristics (Newham et al, 1987). If these indices represent the start of the sequence of events leading to fibre degeneration (Jones and Round, 1990), then the training adaptation may be such that the ability to repair initial damage is enhanced (Clarkson and Tremblay, 1988). In a trained/adapted muscle, any damage-induced increase in intracellular [Ca^{2+}] may be a reversible processes, such that local Ca^{2+} influx and the prolonged elevation of intracellular [Ca^{2+}] may be controlled.

1.5.3. Muscle length.

Eccentric exercise-induced changes in the muscle length-tension curve have been postulated (Morgan, 1990), such that the training effect is an increase in the number of sarcomeres connected in series in a muscle fibre. This would enable sarcomeres to remain on the ascending limb of their length tension curve over the range of muscle lengths used in exercise, and possibly avoid any sarcomere instability. This suggestion was experimentally tested using rat skeletal muscle (Lynn and Morgan, 1994), and data presented supported the theory of increased numbers of sarcomeres in series following downhill running training. Evidence to support this theory was presented by Donnelly et al (1995), such that a chronic shift in the muscle length tension curve following eccentric exercise was reported for human skeletal muscle. However, the involvement of chronic changes in the length-tension relationship of skeletal muscle in adaptation to eccentric exercise has not been fully determined.

Clearly, more work on the mechanisms of skeletal muscle adaptation to eccentric exercise is required. It is probable that in humans, adaptation represents a combination of phenomena. Altered muscle fibre recruitment,

enhanced repair mechanisms, an altered muscle length-tension relationship may all contribute to the 'protection' of skeletal muscle to repeated bouts of eccentric exercise. Although speculative, it may be that skeletal muscle trained by eccentric contractions is more resistant to disuse atrophy or myotoxic substances, however, any clinical benefits of 'adaptated muscle' have not been thoroughly investigated.

1.6. Study aims.

Although much work has been directed toward the mechanisms and time course of exercise-induced muscle damage and adaptation, further research may yield explanations for some of the remaining questions. For example, to what extent is secondary degeneration an inflammatory process, and is this dependent on the extent of initial mechanical overload? Is connective tissue disruption a feature of the initial mechanical events associated with exercise-induced muscle damage, and to what extent is connective tissue involved in adaptation to eccentric exercise? The use of a laboratory based exercise model which can reliably induce temporary, repairable muscle damage, and allow the controlled study of muscle degeneration and regeneration processes represents a valuable tool for further studies in this area. High force isokinetic eccentric muscle contractions may represent an exercise paradigm unfamiliar to many individuals, although many sports activities do involve some eccentric muscle contractions. However, identifying damage and adaptation mechanisms, and assessing muscle tissue vulnerability using these exercise models, may help to reveal the nature of muscle damage in more common exercise modalities.

The aims of the present studies were:

1. To develop non-invasive techniques to assess functional impairment of human skeletal muscle following exercise.
2. To further investigate eccentric exercise-induced damage and adaptation in human skeletal muscle using indirect indices of muscle injury.
3. To investigate connective tissue disruption using markers of collagen breakdown following high force eccentric and concentric exercise models.

Chapter 2

Methods.

Proceeding studies (see chapters 3, 4, 5, and 6) used methods detailed in this chapter, although any modifications to the techniques described below are fully detailed in the appropriate chapter. Methods used throughout this thesis are consistent with studies on human skeletal muscle damage. The preceding review of indirect indices of muscle tissue injury (see 1.3.) and indices of collagen metabolism (see 1.4), collectively detail the relevance of the following methods to studies in muscle and connective tissue damage.

This chapter is structured in the following way: Following a description of the methods used in soreness assessment, details of the tests of skeletal muscle function are described. Sample collection procedures are then described, followed by a description of the biochemical methods used in the analysis of these samples.

2.1. Assesment of muscle soreness.

Delayed onset muscle soreness (DOMS) of the knee extensors was assessed using a questionaire incorporating a visual-analogue scale from 1 (normal) to 10 (very, very sore) for six sites on the anterior muscles of the upper leg and two sites on the posterior muscles of the upper leg. Since no soreness was recorded on the two posterior sites throughout a number of studies (e.g. chapter 4), only the 6 locations on the anterior knee extensors were used in subsequent studies. Subjects were required to self-palpate the relaxed muscle and to rate the soreness on a scale of 1 to 10 for each site, and the values were summed for the criterion score. An example of the questionnaire is shown in appendix 1.

2.2. Measurement of muscle function in vivo:

2.2.1. Maximum voluntary isometric contraction force (MVC).

The change in the maximum force developed can be used for quantitative assesment of the changes in the magnitude of muscle injury with time (Faulkner et al, 1993). Therefore, throughout the studies on exercise-induced damage to human skeletal muscle presented in this thesis, measurement of forces produced during maximum isometric contraction have been recorded. Maximum voluntary isometric muscle contraction force at approximately 90 degrees knee flexion was assessed using an adapted chair and strain gauge system. This was used in preference to the Kin-Com

isokinetic dynamometer for measurement of isometric muscle force due to increased signal sampling frequency, the ability to record stimulator output and force recordings in parallel onto one recorder, and an improved ability to isolate the knee extensor muscles. The electrical signal produced from deformation of the strain gauge (sampled at 1KHz) was amplified (MacLab Bridge Amp, AD Instruments, Undercliffe House, Rock-a-Nore Road, Hastings, East Sussex, UK), converted from an analogue signal to a digital signal (MacLab V8s, AD Instruments, UK), and recorded onto a computerised chart recorder (MacLab Chart, AD Intruments, UK). The strain gauge was regularly calibrated using a series of known weights (up to 80 kg), with mV units converted to appropriate values (in Newtons) using MacLab Chart software (AD Instruments, UK).

2.2.2. MVC with superimposed percutaneous electrical myostimulation (MVS).

During a 3 second MVC, a 1 second pulse of percutaneous electrical myostimulation (PES) at 100 Hz was superimposed on the contracting muscle. The PES was applied to the knee extensor muscles using large (15 cm x 20 cm), moistened, felt covered copper electrodes attatched to a fixed current/variable voltage signal generator (Bioscience Type 200, Bioscience, UK), employing an unidirectional 0.5 ms square wave pulse. Stimulation voltage (at 100 Hz) was always sufficient to induce at least 40 % of the subject's MVC on the day of testing, without voluntary effort. This was achieved by calculating 40 % of the MVC for each subject on the day of testing, and increasing the continuously applied voltage (at 100 Hz) until the desired force was obtained. A 2 min recovery period was used between attaining the required force using PES and further testing.

2.2.3. 20:100 Hz force ratio.

Force frequency characteristics of the knee extensor muscles were determined using the strain gauge system and the percutaneous eletctrical myostimulation (PES) protocol described in 2.2.2. The ratio of knee extensor isometric contraction forces (at approximately 90 degrees knee flexion) produced by PES at 20 Hz and 100 Hz stimulation frequencies was used as an index of low-frequency fatigue. Tetanic force at 100 Hz PES was always sufficient to induce at least 40 % of the subjects MVC on the day of testing.

2.3. Sample collection procedures.

Venous blood samples (10-15 ml) were obtained from a suitably prepared antecubital fossa vein, following the University guidelines for safe blood sampling and handling. For the collection of plasma, approximately 3-5 ml was placed in a plastic container containing potassium EDTA (Type KE/4, L.I.P., 111 Dockfield Road, Shipley, West Yorkshire, U.K.) and centrifuged at 3000 rev.min^{-1} (1500 g) for 10 min, thus allowing the plasma layer to be recovered. For the collection of serum, part of the venous blood sample (6-8 ml) was placed into a tube with neutral density beads coated with a coagulation agent (Type Z/10/T, L.I.P., Shipley, UK), and allowed to clott at room temperature for 30 min. This sample was then centrifuged at 1500 g for 10 min, thus allowing the serum layer to be recovered. Serum and plasma samples obtained by these methods were decanted into plastic vials, sealed, and stored at -20 ^{0}C prior to analysis.

First morning, mid-flow urine samples (approximately 20 ml) were collected into sterile containers. Samples were acidified for preservation using 0.2 ml 6M HCl, and stored at -20 ^{0}C prior to analysis.

2.4. Creatine kinase (EC 2.7.3.2) assay.

Serum creatine kinase (CK) activity has been assayed using a diagnostic kit (DG147-K, Sigma, Poole Dorset, UK). This procedure is based on the method adopted by the German Society for Clinical Chemistry (Szasz et al. 1976). The measurement of serum CK has been used to indirectly indicate the loss of integrity of the muscle cell membrane following unaccustomed exercise on the basis of leakage of this 80KD enzyme into the blood.

The enzymatic reactions involved in the assay are :

Creatine Phosphate + ADP -------> Creatine + ATP (enzyme: Creatine Kinase)

ATP + Glucose ---------> ADP + Glucose-6-Phosphate (enzyme: Hexokinase)

Glucose-6-Phosphate + NAD ------> 6-Phosphogluconate + NADH (enzyme: G-6-PDH)

During the final oxidation reaction, an equimolar amount of NAD is reduced to NADH, increasing the absorbance at 340nm. The rate of change in absorbance is directly proportional to CK activity.

The assay was carried out at 30 ^{0}C using a temperature controlled cuvette compartment on a spectrophotometer (DU70, Beckman Instruments, High Wycombe, UK). Briefly, 20 µl of serum was added to 1 ml of reconstituted CK reagent, incubated for 3 min, and the change in absorbance at 340 nm was recorded for a further 2 min. One unit of activity was defined as the amount of enzyme which produces one µmole.l^{-1} of NADH.min^{-1} under assay conditions.

2.5. Lactate dehydrogenase (EC 1.1.1.27) assay.

Serum lactate dehydrogenase (LDH) activity has been assayed using a diagnostic kit (DG1340-UV, Sigma). LDH catalyses the interconversion of lactate and pyruvate, and primary locations of LDH include skeletal muscle, heart, liver, kidney, and brain. LDH activity is significantly elevated during myocardial infarction, with maximum levels reached approximately 48 hours after the onset of pain and persisting for up to 10 days.

The enzymatic reaction of the assay is

Pyruvate +NADH + H^{+} ------> Lactate + NAD^{+} (enzyme: lactate dehydrogenase)

During the reduction of pyruvate, an equimolar amount of NADH is oxidised to NAD. The oxidation of NADH results in a decrease in the absorbance at 340 nm, and the rate of change in absorbance at 340 nm is directly proportional to LDH activity in the sample.

The assay was carried out at 30 ^{0}C using a temperature controlled cuvette compartment on a spectrophotometer (DU70, Beckman Instruments, High Wycombe, UK). Briefly, 50 µl of serum was added to 1.25 ml of the reconstituted reagent, incubated for 30 s, and the change in absorbance at 340 nm was recorded for a further 3 min. One unit of LDH activity was defined as the amount of enzyme required to produce one µmole.l^{-1} of NAD.min^{-1} under assay conditions.

Serum LDH-1, an isoenzyme of lactate dehydrogenase, has also been assayed using a diagnostic kit (DG120-K, Sigma). This particular isoenzyme is also known as alpha-hydroxybutyrate dehydrogenase, and is

predominantly found in type 1 skeletal muscle and cardiac muscle. LDH-1 shows the greatest catalytic activity in the following reaction:

2-Oxobutyrate + NADH ----------> 2-Hydroxybutyrate + NAD (enzyme: LDH-1)

The rate of change in absorbance at 340 nm is directly proportional to the amount of LDH-1 in the sample. The assay was carried out at 30 ^{0}C using a temperature controlled cuvette compartment on a spectrophotometer (DU70, Beckman Instruments). Briefly, 50 µl of serum was added to 1.25 ml of the reconstituted reagent, incubated for 30 s, and the change in absorbance at 340 nm was recorded for a further 3 min. One unit of LDH-1 activity was defined as the amount of enzyme required to produce one µmole.l^{-1} of NAD.min^{-1} under assay conditions.

2.6. Alkaline phosphatase (EC 3.1.3.1) assay.

Serum alkaline phosphatase (ALP) activity has been assayed using a diagnostic kit (DG1245-K, Sigma). ALP is a commonly used marker of bone formation although the sensitivity and specificity of this marker has been questioned (Delmas, 1993). A moderate rise in ALP activity is observed in osteomalacia and rickets, and measurement of serum ALP can be useful in the diagnosis of bone disorders associated with increased osteoblastic activity.

Alkaline Phosphatase catalyses the following reaction :

p-Nitrophenyl Phosphate + H_2O -----> p-Nitrophenol + Phosphate (enzyme: alkaline phosphatase)

Serum ALP hydrolyzes p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate. The hydrolysis occurs at alkaline pH and the p-nitrophenol formed shows an absorbance maximum at 405 nm. The rate of increase in absorbance at 405 nm is directly proportional to ALP activity in the sample. The assay was carried out at 30 ^{0}C using a temperature controlled cuvette compartment on a spectrophotometer (DU70, Beckman Instruments). Briefly, 25 µl of sample was added to 1.5 ml reconstituted reagent, mixed, and the change in absorbance at 405 nm was recorded for a further 3 min. One unit of ALP activity was defined as the amount of enzyme which catalyses the formation of one µmol.l^{-1} p-nitrophenol.min^{-1} under the assay conditions.

2.7. Creatinine assay.

Urinary excretion of creatinine was measured using a commercially available assay kit (555-A, Sigma). Creatinine is the anhydride of creatine, formed primarily in muscle by the irreversible removal of water from creatine phosphate. Linearity of the creatinine assay on the Beckman DU70 spectrophotometer was determined using the supplied standard at different concentrations. Repeated analysis of the same urine sample was used to calculate the assay CV, and the standard curve was repeated each day while performing the assay. The assay was based on the method of Heinegard and Tiderstrom (1973), where colour intensity of the 'Jaffe chromogen', measured at 500 nm before and after acidification, is proportional to creatinine concentration.

Briefly, a 0.3 ml urine sample (diluted to 1 in 10 using distilled water) was added to 3 ml alkaline picrate solution and allowed to stand fo 8 - 10 min, before recording absorbance at 500 nm. To this, 0.1 ml acid reagent was added, mixed, and allowed to stand for 5 min. Again, absorbance at 500 nm was recorded, and the difference between the initial absorbance and final absorbance was used to calculate the creatinine concentration.

2.8. Spectrophotometric methods to determine the urine concentration of imino acids characteristic of collagen.

The molecular formula for a collagen alpha chain is $(X\text{-}Y\text{-}Gly)_{333}$ where X and Y represent amino acids other than glycine (Prockop et al, 1979). The repetition of glycine residues allows for continuity of the primary structure, because this amino acid occupies a restricted space where the three helical alpha chains come together in a triple helix. Approximately one third of the X positions are proline and one third of the Y positions are hydroxyproline. Both proline and hydroxyproline are rigid, cyclic amino acids which limit the rotation of the alpha chain, thereby contributing to the stability of the triple helix. It is the hydroxyl group of hydroxyproline that plays an essential role in stabilizing the collagen triple helix. The majority of hydroxyproline present in mammalian collagen is the 'trans-4' isomer, although all known collagens (especially those in basement membranes) contain a small amount of the 'trans-3' isomer (Prockop et al, 1979). Hydroxylysine (HL) is also chararacteristic of collagen and related glycoproteins, and although much less abundant than HP, hydroxylysine is a

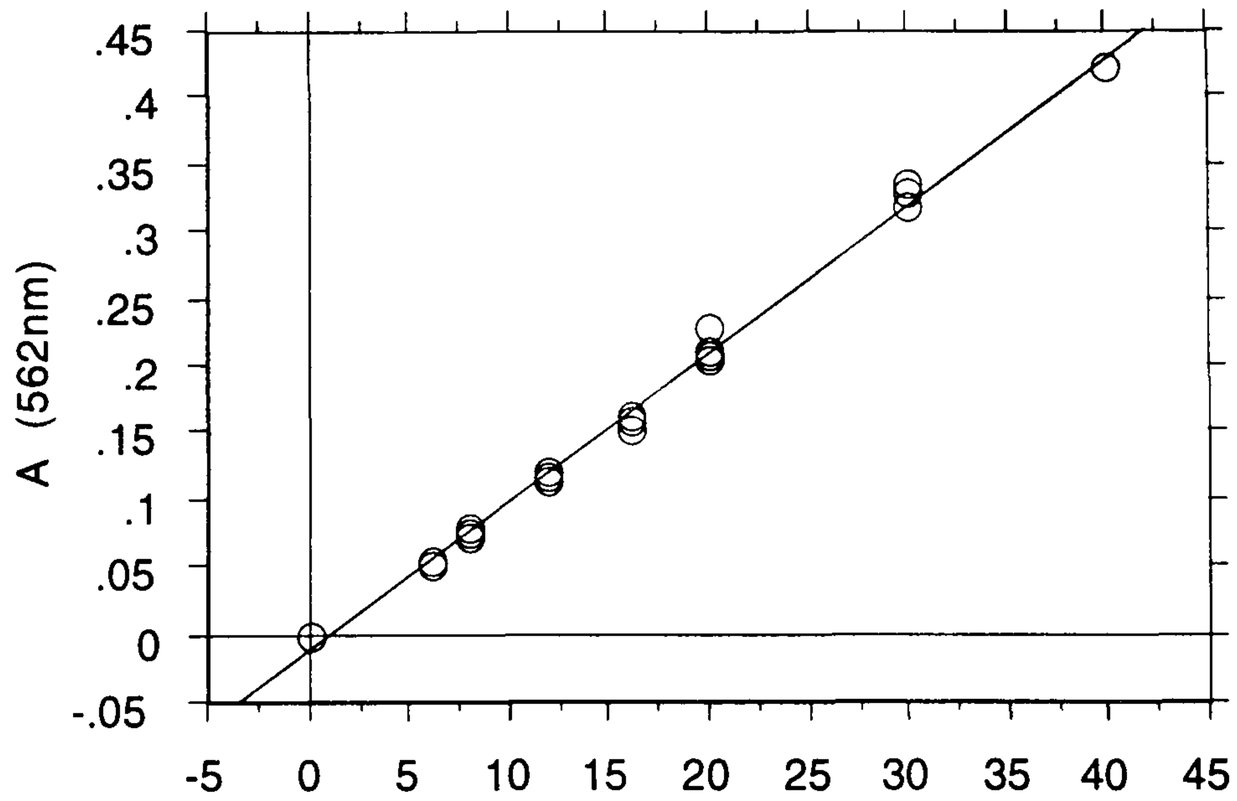
potential marker of collagen degradation. Hydroxylysine is present in part as galactosyl-hydroxylysine and also as glucosyl-galactosylhydroxylysine (Delmas, 1993), and methods to seperate urine hydroxylysine from its glycosylated derivatives have been reported (Bisbee and Kelleher, 1978).

The urinary concentrations of both hydroxyproline and hydroxylysine can be assayed using manual procedures:

2.8.1. Hydroxyproline assay.

A spectrophotometric method for the urinary concentration of hydroxyproline was based on the procedure described by Bergman and Loxley (1970). All chemicals were purchased from Sigma Chemical Company (Poole, Dorset, UK). The buffer required to make up the oxidant solution contained 57.0 g sodium acetate, 37.5 g trisodium citrate, 5.5 g citric acid, and 385 ml isopropanol. This mixture was made up to 1 l using distilled water. A 7 % (w:v) aqueous solution of chloramine-T (N-Chloro-p-toluene sulphonamide sodium salt) was made up daily by mixing 7 g of cloramine-T in 100 ml of distilled water. One volume of the 7 % chloroamine-T solution was mixed with 4 volumes of the buffer on the day of use to make up the oxidant solution. Ehrlick's reagent was prepared by mixing 17.6 g PDAB (p-dimethyl-amino-benzaldehyde) with 40.8 g 60 % perchloric acid. This was made up to 100 ml with isopropanol just prior to use.

Fig 2.8.1. Hydroxyproline standard curve



r=.996

Y = -.013 + .011X

A 20 ml first morning mid-stream urine sample was collected into a sterile universal container and acidified with 0.2 ml 6M HCl. Samples were stored at -20 ^{0}C before duplicated analysis. A 2.5 ml aliquot of defrosted urine was hydrolysed in 2.5 ml 6M HCl at 110 ^{0}C for 6 h. After cooling and centrifugation at 5000 rev.min^{-1} for 10 mins (4500 g), 2 ml of the hydrolysate was neutralised with a lithium hydroxide solution (10 g LiOH, 25 g KNO_3, made up to 250 ml with distilled water) using 5 drops of phenolphthalein (0.25 g phenolphthalein in 150 ml ethanol, made up to 250 ml with distilled water) as a pH indicator. This solution was then made slightly acidic using 1 drop of 6M HCl, and made up to 12.5 ml with distilled water.

A 1 ml aliquot of the prepared hydrolysate was mixed with 2 ml isopropanol and 1 ml of the oxidant solution, mixed and left at room temperature for 4 min. Two ml of the Ehrlick's reagent was added, and the solution was placed in a 60 ^{0}C water bath for 10 min. After cooling for 1 h, the absorbance of the solution was read at 562 nm using a spectrophotometer (CE 1010, Cecil Instruments, Cambridge, UK.).

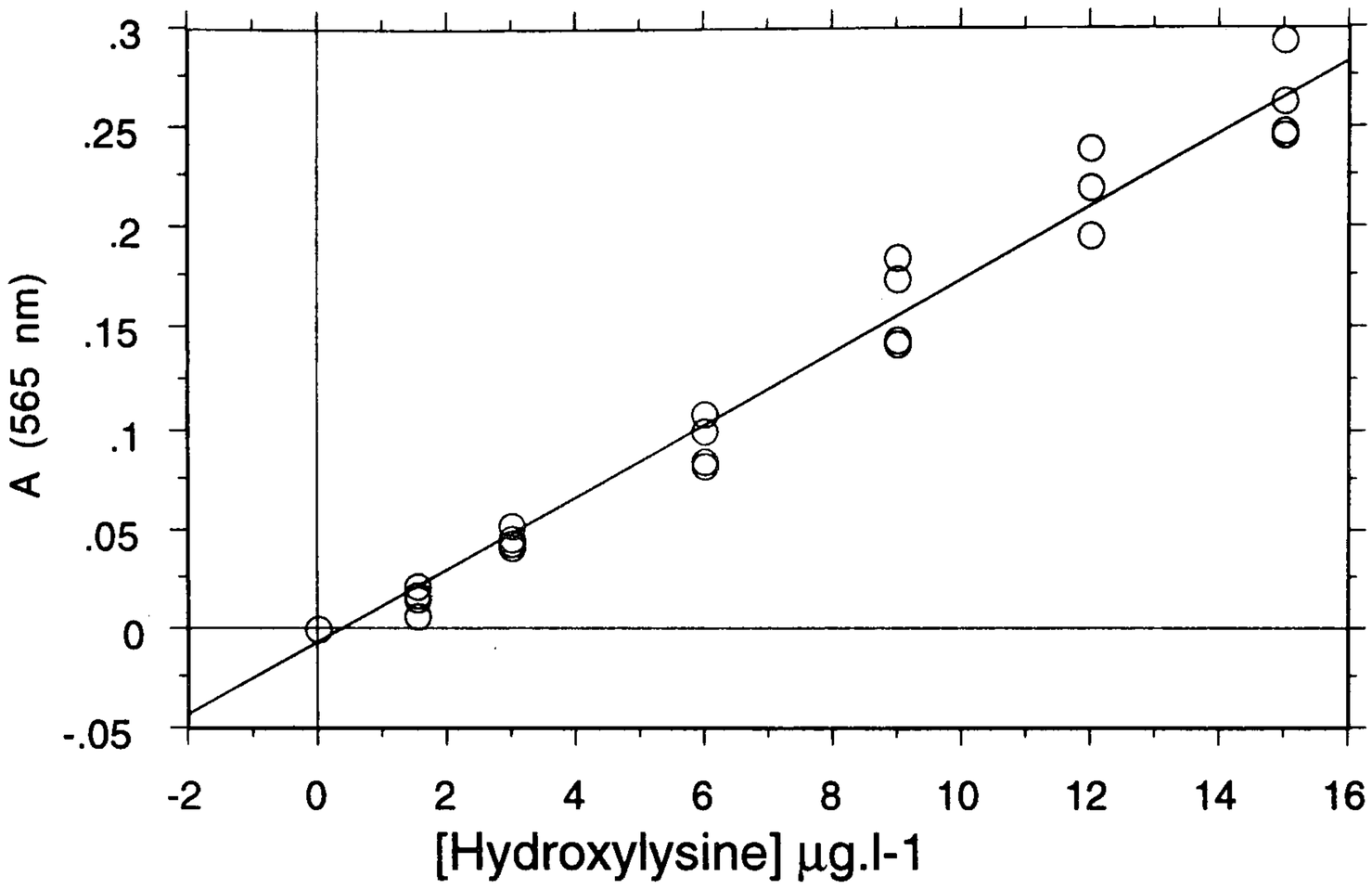
A 'trans-4-hydroxyl-l-proline' standard (Sigma, Poole Dorset, UK) was used to prepare a standard curve. Standards in the range of 6-40 μg.l^{-1} were made using a 200 μg.l^{-1} stock solution, with a 'blank' made up by substituting the standard with distilled water (see fig 2.8.1).

2.8.2. Hydroxylysine assay.

Periodate oxidation of hydroxylysine gives glutamic semialdehyde in equilibrium with Δ-pyrroline-5 carboxylic acid, which in turn is oxidised to form a chromogen with Ehrlich's reagent (Blumenkrantz and Asboe-Hansen, 1975). This reaction forms the basis of the manual assay for determining urinary hydroxylysine concentration.

A 20ml mid-stream first morning sample was acidified with 0.2ml 6M HCl and frozen at -20 ^{0}C prior to analysis. All analyses were carried out in duplicate or triplicate. Aliquots of defrosted urine (1 ml) were precipitated with 5 ml acetone and refrigerated (<6 degrees C) for 30 min. Following centrifugation at 2000 rpm for 15 mins, the supernatant was removed by vacuum suction and discarded. After drying in air, the precipitate was hydrolysed in 1 ml 6M HCl at 115 ^{0}C for 18 hours. Samples were air dryed and reconstitued in 4 ml citrate phosphate buffer (0.15M citric acid : 0.6M dibasic sodium phosphate, 1 : 2.25, pH 7.0)

Fig. 2.8.2. Hydroxylysine standard curve



r=.996

Y=-.013+.011X

A .25 ml aliquot of the prepared sample was mixed with .6 ml sodium metaperiodate (0.0015M in diluted citrate phosphate buffer, 1:3 in distilled water) and allowed to stand at room temperature for 20 mins, protected from the light. To this, 0.17 ml of Ehrlich's reagent (2 g p-dimethyl-amino-benzaldehyde dissolved in 7.5 ml perchloric acid, with 7.5 ml isopropanol) was added, mixed, and allowed to stand for a further 10 min. Absorbance was read at 565 nm using a spectrophotometer (CE 1010, Cecil Instruments, Cambridge, UK.). A standard curve was prepared using δ-hydroxylysine (2,6-Diamino-5-hydroxyhexanoic acid) at 1.5 - 15 $\mu g.l^{-1}$ concentrations.

2.8.3. Measurement of hydroxyproline in plamsa.

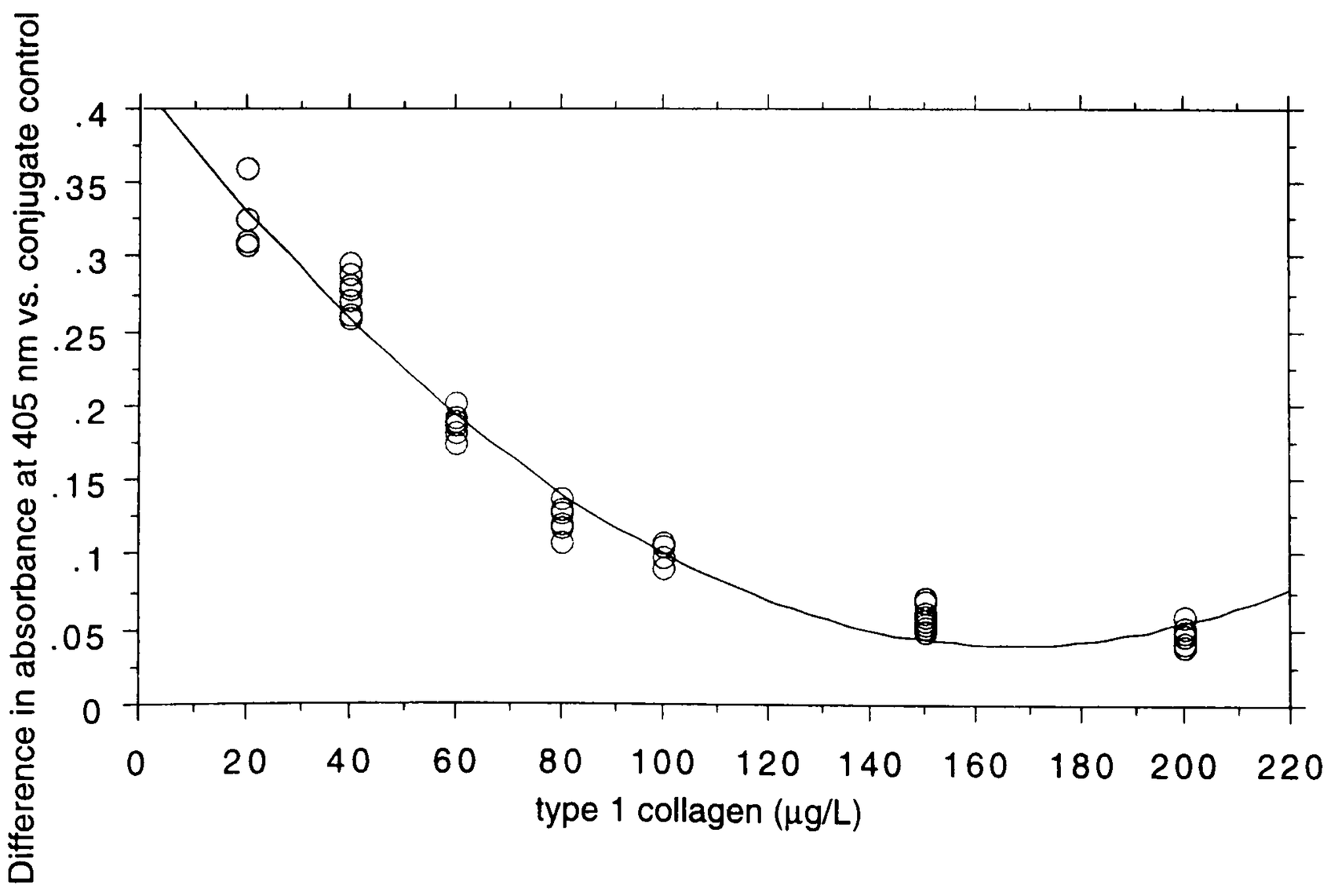
Plasma hydroxyproline was measured using the combined methods described by Murguia et al (1988) and Bergman and Loxley (1963). A 0.5 ml aliquot of plasma was mixed with 0.5 ml 8 % trichloro-acetic acid, vortexed for 1 min, and then centrifuged at 1500 g for 5 min. A 0.375 ml aliquot of the supernatant was mixed with 0.375 ml 6MHCl, sealed in plastic vials, and hydrolysed at 110 degrees C for 15 hours. Following evaporation, samples were reconstituted with 0.5 ml distilled water, and assayed for hydroxyproline content. A 0.4 ml aliquot of the prepared hydrolysate was

mixed with 0.4 ml oxidant solution (see 2.8.1.), and 0.8 ml isopropanol, and allowed to stand for 4 min. To this, 0.8 ml Ehrlick's reagent (see 2.8.1.) was added and the solution was placed in a 60 ^{0}C water bath for 10 min. After cooling for 1 hour, the absorbance of the solution was then read at 562 nm using a spectrophotometer (CE 1010, Cecil Instruments).

2.9. Inhibition enzyme linked immunoassay (ELISA) for serum type 1 collagen.

An inhibition ELISA method for the measurement of type 1 collagen in human serum, based on the method of Rennard et al (1980), was used to indicate breakdown of connective tissue following concentric and eccentric exercise. In this method, plastic microtiter plates are coated with purified antigen, and following incubation of antigen and antibody mixtures, any remaining unbound antibody is allowed to bind to the coated plate. The amount of bound antibody is determined by incubation with a secondary antibody which is covalently linked to an enzyme. Bound enzyme is assessed by addition of a suitable substrate, and the amount of antigen in the initial incubation is estimated from its ability to inhibit the binding of the first antibody to the antigen coated well.

Fig. 2.9. Polynomial regression standard curve for serum type 1 collagen concentration



Flat bottomed 96 well polystyrene microtiter plates (Costar Ltd., Bucks, UK) were coated with purified antigen (human placental type 1 collagen, Biogenesis, Dorset, UK) at a concentration of 0.15 $\mu g.ml^{-1}$ in carbonate buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate, 3 mM sodium azide, pH 9.6), and allowed to adsorb for 48 hours at <4 ^0C in a humidified chamber. Quantities of antigen and unknowns were incubated overnight with antibody (polyclonal rabbit antibody to human collagen type 1, Biogenesis, Dorset, UK), at <4 ^0C in sealed eppendorf containers. Antibody dilution was 1:2000 in de-ionised, double distilled water, and cross reactivity with other collagens was quoted at less than 3 %. Plates were washed with PBS + Tween (0.02 M sodium phosphate, 0.15 M sodium chloride, with 0.05 % Tween 20) using a manual procedure of 5 x 200 µl with a Titertek multichannel pipettte (Eflab, Finland). Antigen/antibody and unknown/antibody preparations were transferred to the coated wells (200 µl into each well in triplicate), and unbound antibody was allowed to bind coated antigen overnight at <4 ^0C. Plates were then washed as before, and 200 µl of 1:750 goat anti-rabbit IgG peroxidase conjugate (Sigma, Dorset, UK) was added to each well and incubated at <4 ^0C for 2 hours. Following another washing step, enzyme substrate (2,2-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS)) was added to each well (1 tablet in 50 ml 0.05M phosphate-citrate buffer, pH 5.0, with 12.5 µl of 30 % hydrogen peroxide added), and the reaction was allowed to proceed for 30 min. at room temperature. Absorbance was measured at 405 nm using an automated plate reader (Multiscan MS, LabSystems, Finland). Plates contained control columns such that column 1 contained antibody, conjugate, and substrate, but no coating; column 2 contained coating, conjugate, and substrate, but no antibody; column 3 contained coating, antibody, and substrate, but no conjugate; and column 4 contained coating, antibody, and conjugate, but no substrate.

Rennard et al (1980) reported a sigmoidal standard curve for serum type 1 collagen concentration using the same method described here. The second order polynomial regression (see Fig. 2.9.) provided the closest 'line of best fit' to the standard curve data (r=.969), and since values were within the 40 - 120 $\mu g.l^{-1}$ range, this curve provided a suitable means to predict concentrations in serum from the absorbance readings.

2.10. Analysis of Pyridinoline and Deoxypyridinoline using high pressure liquid chromatography (HPLC).

Covalent intermolecular cross-linking in collagen and elastin partially confer the physical and chemical properties of these connective tissue proteins. Pyridinoline (PYD), a naturally fluorescent compound first isolated from bovine achilles tendon by Fujimoto et al. (1978), is a mature cross linking compound found in collagen. It appeared to be a stable amino acid derivative formed by lysyl oxidase during collagen maturation. Using information based on the structure and function of pyridinoline, Fujimoto et al. (1978) postulated it's formation from the condensation of one hydroxylysine and two hydroxylysine derived aldehydes (hydroxyallysine). A derivative of pyridinoline, involving a lysine rather than hydroxylysine residue in the helix has also been identified and named deoxypyridinolne (DPYD) (Ogawa et al. 1982). PYD and DPYD have also been termed hydroxylysinepyridinoline and lysylpyridinoline respectively (Uebelhart et al. 1990).

Urinary PYD concentrations have been measured using HPLC and ELISA techniques. James et al. (1990) reported an isocratic HPLC method for PYD and DPYD quantification in urine, and these authors detailed the effects of pH and buffering salts on the resolution and peak areas of PYD and DPYD using optimised fluorescence monitoring conditions. The use of automated HPLC systems have also been reported (Pratt et al. 1992). System automation may give improved reproducibility over the manual procedure, and be suitable for routine clinical assessment of large numbers of samples. Critcisms of the HPLC technique have been levelled at the extensive solid phase extraction procedures required in sample preparation, and therefore ELISA techniques have been further developed.

2.10.1. Pyridinoline analysis using System Gold (Beckman Instuments, High Wycombe, U.K.).

A method similar to that reported by Eyre et al. (1984), with modifications based on information from Abiatti et al. (1993), was originally adopted for HPLC determination of PYD. All HPLC grade chemicals were purchased from BDH (Poole, Dorset, UK.) or Sigma (Dorset, UK.), and all solvents were degassed using helium. A PYD standard was kindly supplied by Dr. S. Robins (Rowett Institute, Bucksburn, Aberdeen, U.K.).

To a 20 ml mid stream first morning urine sample, 0.2 ml 6 mol/l HCl was added, and samples were stored at -20 ^{0}C until analysis. Aliquots of defrosted urine (500 µl) were hydrolysed in 500 µl concentrated HCl at 110 ^{0}C overnight in sealed glass containers. Two ml of butanol, 0.5 ml glacial acetic acid, and 0.5 ml CF1 cellulose (50 g.l^{-1} in a mixture of n-butanol, acetic acid, and water [4:1:1 by volume]), was added to the hydrolysed sample and mixed thouroughly. The sample was then applied to a column containing 300 mg of cellulose and eluted with 10 ml of water. The eluent was evaporated at 110 ^{0}C and the residue reconstituted with 100 µl of 10 g.l^{-1} n-heptaflourobutyric acid (HFBA).

These prepared samples were filtered and analysed using a dual pump System Gold HPLC (Beckman Instruments, High Wycombe, U.K.) attached to a Shimadzu RF535 Fluorescence Detector (Dyson Instruments, UK.). Standard pump priming procedures were carried out to remove possible contamination from the delivery lines and operating pumps. Initial use of the column required a 'settling in' period which necessitated pumping the storeage solvent (70:30 Acetonitrile:Water, v:v)though the column for approximately 2 hours at a 1 ml.min^{-1} flow rate. Flow rate for each pump was checked over a 1 hour period, accuracy for both pumps was >95%. A mobile phase of 15:85 Acetonitrile:Water (v:v) was prepared and a linear gradient of 1 hour duration (at 1 ml.min.$^{-1}$) was required to change the storeage solution to the assay mobile phase. Following this changeover period, detector signal was monitored for a further 30 mins. to indicate stability and consistency of the eluant.

Prepared urine samples were diluted to 20% with the mobile phase, and loaded into a 20 µl sample loop prior to injection using a manual procedure. A 0.5 ml.min^{-1} flow rate, and a 25 cm x 4.6 mm S5 ODS 2 column (Phase Separations, Clwyd, UK.) were used thoughout analysis. Fluorescence was monitored using an excitation frequency of 297 nm with emission at 400 nm. Total run time for each sample was 15 mins., with samples run in duplicate. A calibrator was run at regular intervals (every 15 samples), and within-run repeatability was regularly monitored.

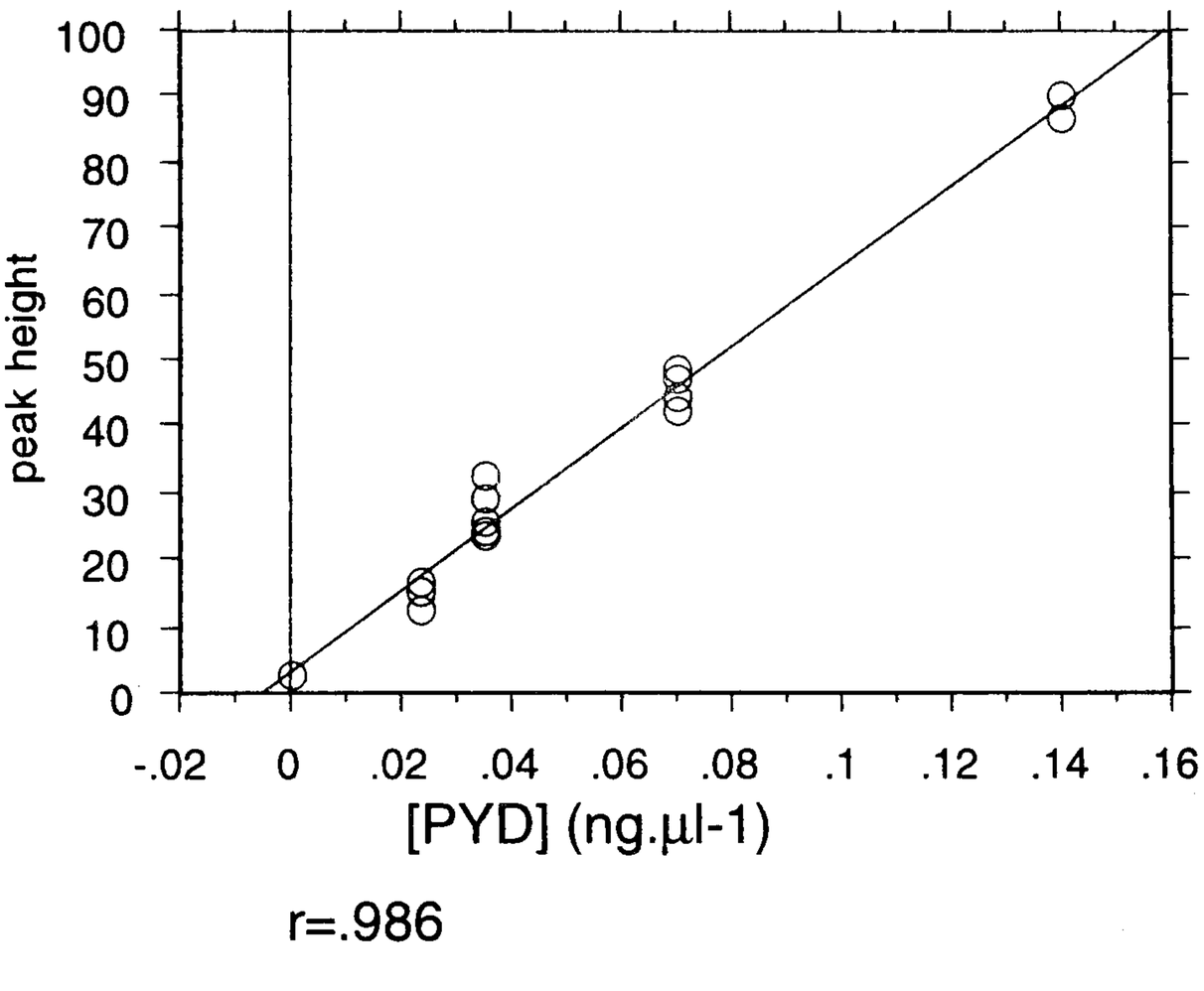
The System Gold was operated using a manual injector accompanied by manual detector activation, and compatibility problems arose with the detector and System Gold software. The system was not suitable for the analysis of large numbers of samples, particularly when cleaning routines were run between samples. Regular flushing of the column with the storage solvent was required to maintain the performance of the assay. Also, regular washes with both methanol and water were required to maintain the

performance characteristics of the column. Despite these measures, detection could be further improved by changing the mobile phase and flow conditions, based on advice from Dr. I. James (St. Mary's Hospital, London, U.K.).

2.10.2. Pyridinoline analysis using the Perkin Elmer system (Norwalk, Cnn., U.S.A.).

The Perkin Elmer (Norwalk, Cnn., U.S.A.) was used in preference to the System Gold because of the convenience of an auto-sampler, compatability between detector and system software, and larger memory storage capability. A method similar to that reported by James et al (1990) was adopted for HPLC determination of the urinary concentration of PYD. Standards of PYD (7.02 $\mu g.ml^{-1}$) and DPYD (2.36 $\mu g.ml^{-1}$) were obtained from Metra Biosystems (Wheatley, Oxfordshire, U.K.). All other chemicals (HPLC grade where appropriate) were obtained from Sigma (Dorset, U.K.) or BDH (Dorset, U.K.).

Fig. 2.10.2. Pyridinoline standard curve



r=.986

Y = 3.266 + 609.236 X

To a 20 ml mid-stream first morning urine sample, 0.2 ml 6 M HCl was added, and the sample was stored at either -20 ^{0}C or -80 ^{0}C until analysis. Aliquots of defrosted urine (1 ml) were hydrolysed in 1 ml 6 M HCl at 110 ^{0}C for 10 hours in a sealed glass container. The hydrolysed sample was mixed with 500 µl of a cellulose suspension (10% w:v CF1 cellulose in butanolic wash [4:1:1 butan-1-ol: glacial acetic acid: water]) and vortexed for 1 min. To

this, 2.5 ml of a butanol: glacial acetic acid solution (4:1 butan-1-ol: glacial acetic acid) was added, and vortexed for another 1 min. This sample mixture was then applied to a prepared cellulose collumn. Cellulose extraction collumns were prepared using a CF1 cellulose suspension (600 mg of cellulose in approximately 8 ml water per sample) and made in 10 ml syringe barrels plugged with a small piece of cotton wool. Cellulose in the suspension was allowed to settle in the collumn before draining the water in an attempt to ensure an equal bed volume. The samples were eluted with 20 ml distilled water, and the eluant was evaporated on hot plates (75 ^{0}C) placed in a fume extraction cupboard (for approximately 3 hours). The remaining residue was reconstituted with 200 µl of the HPLC assay mobile phase, and stored refrigerated prior to analysis. These refrigerated samples were stored for no longer than 48 hours before HPLC analysis.

Prepared samples were analysed isocratically using a Perkin Elmer HPLC system (Norwalk, Cnn., USA). The system consisted of a Binary LC pump, LS40 fluorescence detector, and ISS sample processor. Assay mobile phase was 25 mM sodium formate: 5 mM octanesulphonic acid: 1 mM ethylene diamine-tetracetic acid (EDTA), in 17.5 % methanol. Mobile phase pH was adjusted to 3.5 using glacial acetic acid, and de-gassed using helium. Samples were separated on a 5 µm octadecylsilane reversed phase column (250 mm x 4.6 mm), protected by a 1 cm disposable guard cartridge, obtained from Phase Separations (Clwyd, UK), using a flow rate of 1 ml.min^{-1}. Detection was by fluorescence using an excitation wavelength of 295 nm and an emission wavelength of 400 nm.

All biochemical assay coefficient of variation data is detailed in table 2.1.

ASSAY	MEDIUM	METHOD	NUMBER	CV*
creatine kinase	serum	spectrophotometric (kinetic at 340nm)	n=8	9.1 %
lactate dehydrogenase	serum	spectrophotometric (kinetic at 340nm)	n=8	7.3 %
α-hydroxybutyrate dehydrogenase	serum	spectrophotometric (kinetic at 340nm)	n=8	6.9 %
alkaline phosphatase	serum	spectrophotometric (kinetic at 405nm)	n=8	7.0 %
creatinine	urine	spectrophotometric (end point at 500nm)	n=10	5.4 %
hydroxyproline	urine	spectrophotometric (562nm)	n=10	5.9 %
hydroxylysine	urine	spectrophotometric (565nm)	n=10	8.0 %
hydroxyproline	plasma	spectrophotometric (562nm)	n=8	4.7 %
type 1 collagen	serum	ELISA (absorbance at 405nm)	n=8	9.8 %
pyridinoline	urine	HPLC (fluorescence at ex.295nm, em.400nm)	n=10	7.6 %

* CV = (SD / MEAN) x 100

Table 2.1. Coefficient of variation values for biochemical assays.

Chapter 3.

Changes in human skeletal muscle contractile function following stimulated eccentric muscle contractions.

Parts of this study have been published as conference proceedings, and as a full paper :

Brown S.J., Child R.B., Donnelly A.E., Saxton J.M., Day S.H. (1995) Effects of stimulated eccentric exercise on indices of human muscle contractility. Journal of Physiology 483,121P.

Brown S.J., Child R.B., Day S.H., Donnelly A.E., Saxton J.M. (1996) Changes in human skeletal muscle contractile function following stimulated eccentric exercise. European Journal of Applied Physiology and Occupational Physiology 72:515-521.

3.1. Abstract.

Indices of human skeletal muscle contractile function were examined in 9 subjects for up to 9 days following a single bout of stimulated eccentric exercise. Eccentric muscle actions of the knee extensor muscles were evoked by percutaneous electrical myostimulation (PES). Delayed onset muscle soreness (DOMS), elevated serum Creatine Kinase activity, chronic force loss, and a decline in the 20:100 ratio were observed in the days post exercise. The exercised knee extensor muscles demonstrated an impaired ability to respond to PES. This was evident by an increased time delay between the start of 100 Hz PES and the onset of contraction immediately post exercise (mean, +/-SD, 22.3 +/-15.9%, $P<0.01$) and 3 days post exercise (14.9 +/-18.1%, $P<0.05$). Muscle relaxation rates appeared unaffected by the eccentric exercise protocol, where the muscle showed no differences in the time between the end of PES and the onset of relaxation ($P>0.05$). During the days following the exercise bout, no significant differences were observed in the time between the start of contraction and attainment of 70 % of the mean tetanic force following a single 1 s pulse of PES. Similarly, no significant differences were observed in the time between the start of relaxation and attainment of 70% of the total relaxation over the same time points. The increased delay in excitation-contraction coupling observed immediately post exercise and 3 days after the exercise bout, may reflect a damage induced delay in action potential propagation. Muscle relaxation rates post exercise remained unchanged, which may indicate normal functioning of the sarcoplasmic reticulum, suggesting this was not the site of failure in excitation-contraction coupling.

3.2. Introduction.

A theoretical model of exercise-induced muscle damage cites membrane disruption and disturbed Ca^{2+} homeostasis as causative mechanisms in the etiology of muscle fibre necrosis and secondary degradation (see 1.2.1.). A fully functional sarcoplasmic reticulum (SR) regulates intramuscular Ca^{2+}, and failure of the integrity of the SR, with a subsequent inability to regulate intracellular free [Ca^{2+}], may prolong the elevated Ca^{2+} concentration (Byrd, 1991). This may present an environment which can stimulate Ca^{2+} sensitive autolytic processes which may be involved in myofibril degradation. It has been suggested that chronic low-frequency fatigue (see 1.3.3.) following unaccustomed eccentric exercise

(Jones, 1981) indicated structural damage to the T tubular network in preference to the prolonged alteration of the kinetics of troponin and calcium binding. However, Friden (1984) has suggested that SR disruption following high force eccentric exercise is unlikely, since such damage would manifest itself in contraction clots visible in muscle biopsy specimens. Other authors (Warren et al, 1993) have also hypothesized that high force eccentric muscle contractions cause structural damage to the sarcolemma, with a subsequent loss of normal ion distribution across the membrane.

Voluntary eccentric exercise models have been used to induce damage within the contracting musculature (Newham et al, 1983a; Clarkson et al, 1992; Rodenburg et al, 1993), however, controlling the mass of muscle recruited using percutaneous electrical myostimulation (PES) may remove effects of central fatigue, motivation, and differing motor unit recruitment patterns during a bout of exercise. The PES models consistently evoke contraction within the same muscle mass, and further help to standardise a submaximal exercise protocol within a subject population. Clarkson et al (1992) have measured functional impairment of skeletal muscle following eccentric muscle actions, and these authors have demonstrated a progressive recovery of maximal isometric force following the bout. Newham et al (1987) have measured the ratio of forces produced using 20 and 100 Hz stimulation frequencies post- eccentric exercise, and these authors have also demonstrated a progressive recovery following the exercise. These measures of muscle function, and new indices of skeletal muscle contraction and relaxation (in particular the temporal dissociation between application of an electrical stimulus and contraction/relaxation) were investigated in the present study. The indices of human skeletal muscle contractility were used to assess functional impairment following a single bout of stimulated eccentric exercise.

3.3. Methods.

3.3.1. Subjects.

Nine volunteers (five male, four female, age range 18-35 years) signed written informed consent forms prior to participation. University of Wolverhampton Ethical Committee approval was obtained for this study, and each subject attended the laboratory for a familiarisation session.

3.3.2. Exercise Bout.

Subjects performed one bout of 70 electrically stimulated single leg knee extensor eccentric muscle actions, at an angular velocity of 1.05 rad.s^{-1} through a 1.57 rad. range of motion. The exercise bout was performed on a randomly assigned leg using a Kin-Com II isokinetic dynamometer (Chattecx, TN., USA). Initially subjects performed a single isometric maximum voluntary contraction (MVC) of the knee extensors, at a knee flexion angle of 1.57 rad., when seated on the isokinetic dynamometer. The maximum force achieved was recorded. PES of the knee extensors was evoked using a PES protocol (see 2.2.2.), and PES was sufficient to induce 50 % of the MVC without voluntary effort.

3.3.3. Force Data Collection.

Measures of muscle function were obtained from each subject using equipment previously described (see 2.2.). Force measurements were collected before, immediately after, and on days 1, 2, 3, 7 and 9 following the bout. Duplicate measures of muscle function were collected in a randomised order, and consisted of :

1. knee extension maximum isometric voluntary contraction force at a knee flexion angle of approximately 1.57 rad. (MVC);
2. MVC with superimposed myostimulation was obtained by applying a 1 s pulse of a 100 Hz stimuli during a 3 s MVC at a voltage sufficient to induce at least 50 % of the subject's MVC on the day of testing (MVS). The MVS technique was used to possibly indicate any unconscious inhibition of fibre recruitment during the performance of an MVC. The MVS values may more clearly demonstrate the force generating capability of the exercised muscle;
3. the mean tetanic force produced during a 1 s pulse of 100 Hz PES;
4. the mean tetanic force produced during a 1 s pulse of 20 Hz PES;
5. the time difference between activation of stimulation and the onset of contraction and onset of relaxation, obtained from the strain gauge recording when obtaining 3 and 4 above. Contraction was assumed to have started when the recorded force was 2 standard deviations above the mean residual baseline. Relaxation was assumed to have started when the mean tetanic contraction force decreased by 2 standard deviations. Mean values and corresponding SD values were obtained by statistical analysis of 300 data points (equivalent to 0.3 s) of the baseline and tetanic force, using MacLab Chart v3.3.3 software (AD Instruments, Hastings, East Sussex, UK.). It was

assumed that values exceeding 2 SD values of a calculated mean represented data which was outside the expected random variation.

6. the time difference between the onset of contraction and attainment of 70 % of the mean tetanic force, and the time difference between the onset of relaxation and attainment of 70 % total relaxation. The 70 % criteria represented an attempt to quantify the majority of both contraction and relaxation time courses.

Measures 5 and 6 above may give an indication of any temporal disturbances in muscle excitation contraction coupling.

3.3.4. Indices of muscle damage.

Delayed onset muscle soreness (DOMS) was assessed daily by a questionnaire incorporating a total of eight sites: six sites on the anterior muscles of the upper leg, and two sites on the posterior muscles of the upper leg (see 2.1.).

A 10 ml venous blood sample was collected from the subject's antecubital fossa (see 2.3.) pre exercise, and on days 1,2,3,7 and 9 following the bout. Blood samples were allowed to clot at room temperature for 30 min prior to centrifugation at 1500 g for 10 min, thus allowing the serum layer to be recovered. Serum samples were stored at -20 degrees C prior to analysis, in duplicate, for Creatine Kinase (CK) activity using a diagnostic kit (see 2.4.).

3.3.5. Statistics.

Parametric data were analysed using repeated measures analysis of variance (ANOVA) with post hoc analysis using Duncan's multiple range test. Where stated, paired student t tests were carried out. Soreness data were analysed using a Wilcoxon test for matched pairs.

3.4. Results.

Examples of the strain gauge and stimulator output are detailed in figure 1a and figure 1b. Figure 1a denotes a typical force recording using 100 Hz PES and figure 1b denotes the typical force recording using 20 Hz PES. Complete tetanus did not occur during 20 Hz stimulation and some muscle tremor existed during baseline recording and with 100 Hz

stimulation. This variability in strain gauge output necessitated the use of the 2 SD's criterion as an indicator of the onset of contraction and relaxation.

Delayed onset muscle soreness increased the day after exercise ($P<0.05$, Wilcoxon test) and peaked on day 3 (Table 1). Soreness remained elevated above baseline until day 7 ($P<0.05$, Wilcoxon test) but returned to baseline values by day 8. Serum CK activity (Table 1) increased in days subsequent to the exercise bout ($P<0.01$, repeated measures ANOVA), with the highest recorded mean value on day 3 post exercise. Pre exercise, the mean (+/- SD) MVC and MVS values were 360 N (+/- 147) and 356 N (+/- 159) respectively, and these were not significantly different ($P>0.05$, t test). Both MVC and MVS were reduced after exercise ($P<0.01$, repeated measures ANOVA) with maximum force loss 3 days after exercise (Fig. 2a). Immediately following the exercise bout, the mean decline in MVC was 40.9%, and on day 3 post exercise a 54% loss of force was recorded. The MVS technique consistently increased the force generation by approximately 10 % post exercise, and this difference was significant on days 1,2, and 3 post exercise ($P<0.05$, t test).

The ratio of forces produced at 20 Hz and 100 Hz stimulation frequencies (20:100 ratio, data shown in Fig. 2b), was reduced after exercise ($P<0.01$, ANOVA) and showed a trend toward recovery in days following the bout. Table 2 shows the mean latency values for the onset of contraction and the onset of relaxation using both the 100 Hz and the 20 Hz frequencies. Fig. 3a. shows the relative time delay, from pre exercise, between the start of stimulation and the onset of contraction. Following a 1s pulse of 100 Hz PES, an increase in the time between the start of stimulation and the onset of contraction was observed immediately after exercise ($P<0.01$, paired t test between pre exercise and post exercise), and on day 3 ($P<0.05$, paired t test between pre exercise and day 3 post exercise). A similar trend was observed following a 1s pulse of 20 Hz PES, although these differences were not significant. No significant differences were observed in the time between the end of PES and the onset of relaxation (data shown in Fig. 3b) following both 1s of 100 Hz and 1s of 20 Hz stimulation.

No significant changes were observed in the time between the onset of contraction and attainment of 70% of the total force produced during a 1s pulse of 100 Hz stimulation, or in the time between the onset of relaxation and that taken to reach 70% of the total relaxation from the same stimulus. Similarly, no significant changes were observed in these measures during a 1s pulse of 20 Hz stimulation.

	PRE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 9
DOMS	8 (8-8)	ø 19 (12-26)	ø 32 (21-43)	ø 33 (18-48)	ø 27 (13-41)	ø 18 (10-28)	ø 15 (9-21)	ø 12 (8-15)	8 (8-9)
Serum CK activity $IU.l^{-1}$	87 (43)	247 (1479)	** 6816 (3951)	** 12540 (7335)	—	—	—	* 4281 (4818)	825 (971)

Table 3.1. Upper leg muscle soreness [DOMS, median (range)], and serum creatine kinase (CK) activity following a single bout of stimulated eccentric exercise (mean +/- SD). ø=P<0.05, Wilcoxon test; *=P<0.05 Duncan post hoc following repeated measures ANOVA; **=P<0.01 Duncan post hoc following repeated measures ANOVA.

		PRE	POST	DAY 1	DAY 2	DAY 3	DAY 7	DAY 9
100 Hz	onset of contraction	17.0(2.1)	20.8(3.2)	17.8(2.4)	18.3(3.5)	19.5(2.7)	18.8(2.4)	19.4(3.7)
	onset of relaxation	35.9(4.3)	33.7(6.1)	38.6(9.1)	36.0(6.5)	34.1(6.9)	38.9(14.5)	36.4(6.1)
20 Hz	onset of contraction	17.0(1.8)	18.9(2.5)	16.7(2.1)	17.4(2.0)	18.3(2.7)	17.5(2.7)	18.6(4.3)
	onset of relaxation	57.6(3.5)	59.1(4.4)	58.6(2.4)	58.3(2.3)	58.9(3.2)	59.4(3.8)	56.2(5.0)

Table 3.2. Time delay for the onset of contraction and the onset of relaxation (mean +/- SD values in ms) using 1 s of stimulation with 100 Hz and 20 Hz frequencies, following a single bout of stimulated eccentric exercise.

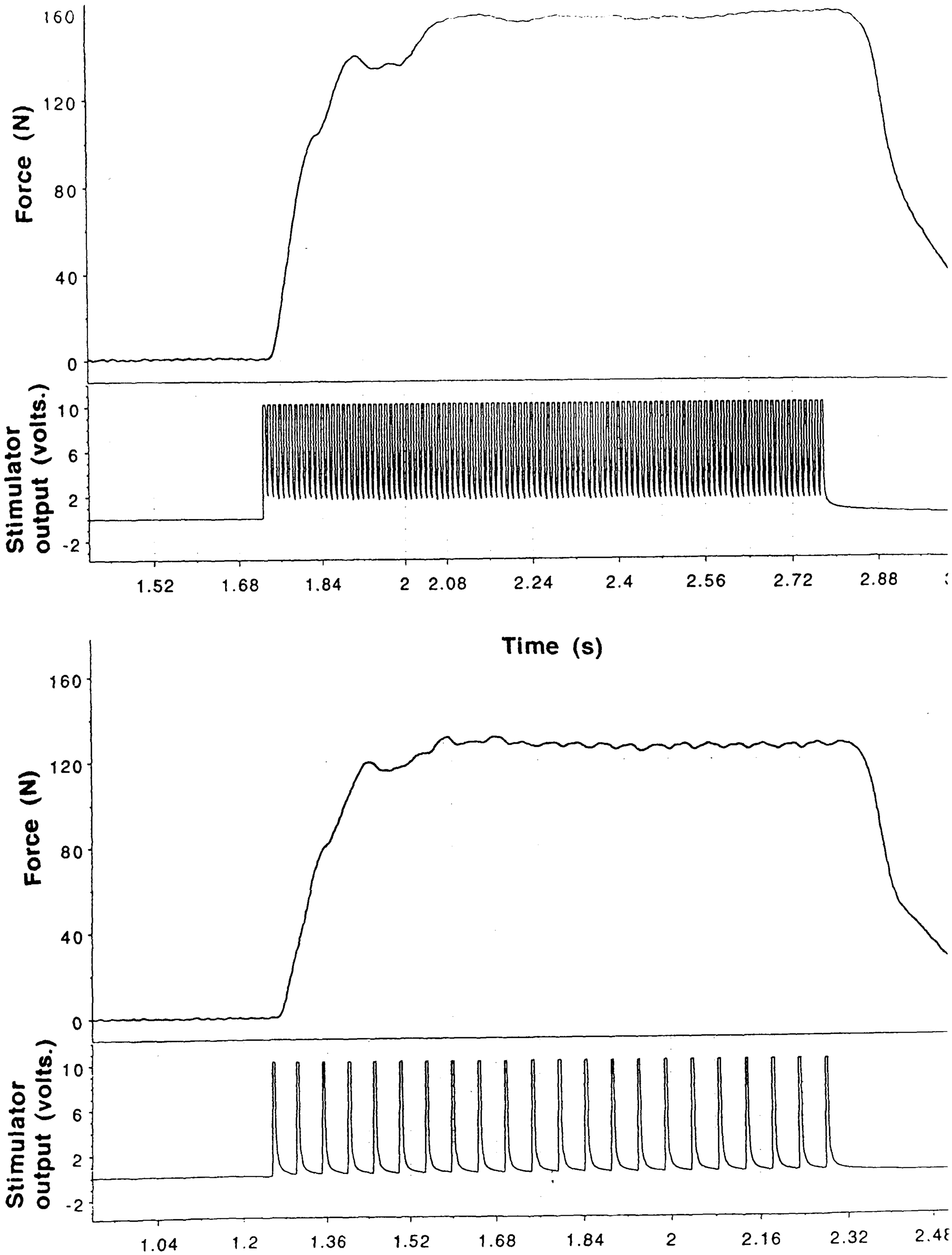


Fig. 3.1. (Top) Typical force and stimulator output with a 100 Hz percutaneous electrical myostimulus. (Bottom) Typical force and stimulator output with a 20 Hz percutaneous electrical myostimulus.

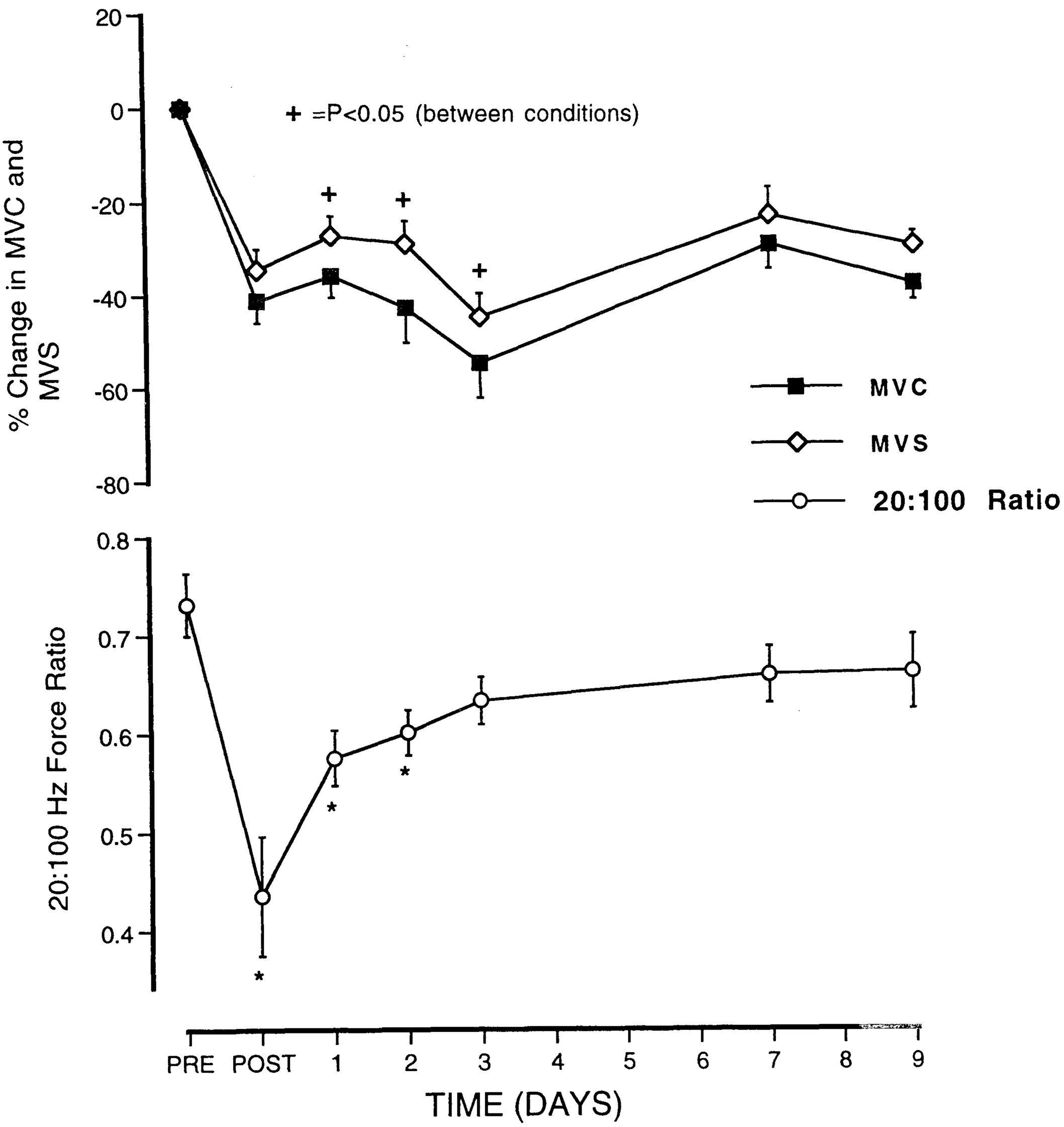


Fig. 3.2. (a and b) Top: % change in maximum voluntary contraction (MVC) and MVC with superimposed myostimulation (MVS) following a single bout of stimulated eccentric exercise (Mean +/- SD). P values using t-test. Bottom: 20:100 Hz stimulated force ratio following a single bout of stimulated eccentric exercise (mean +/- SD). *=P<0.01, Duncan post hoc following repeated measures ANOVA.

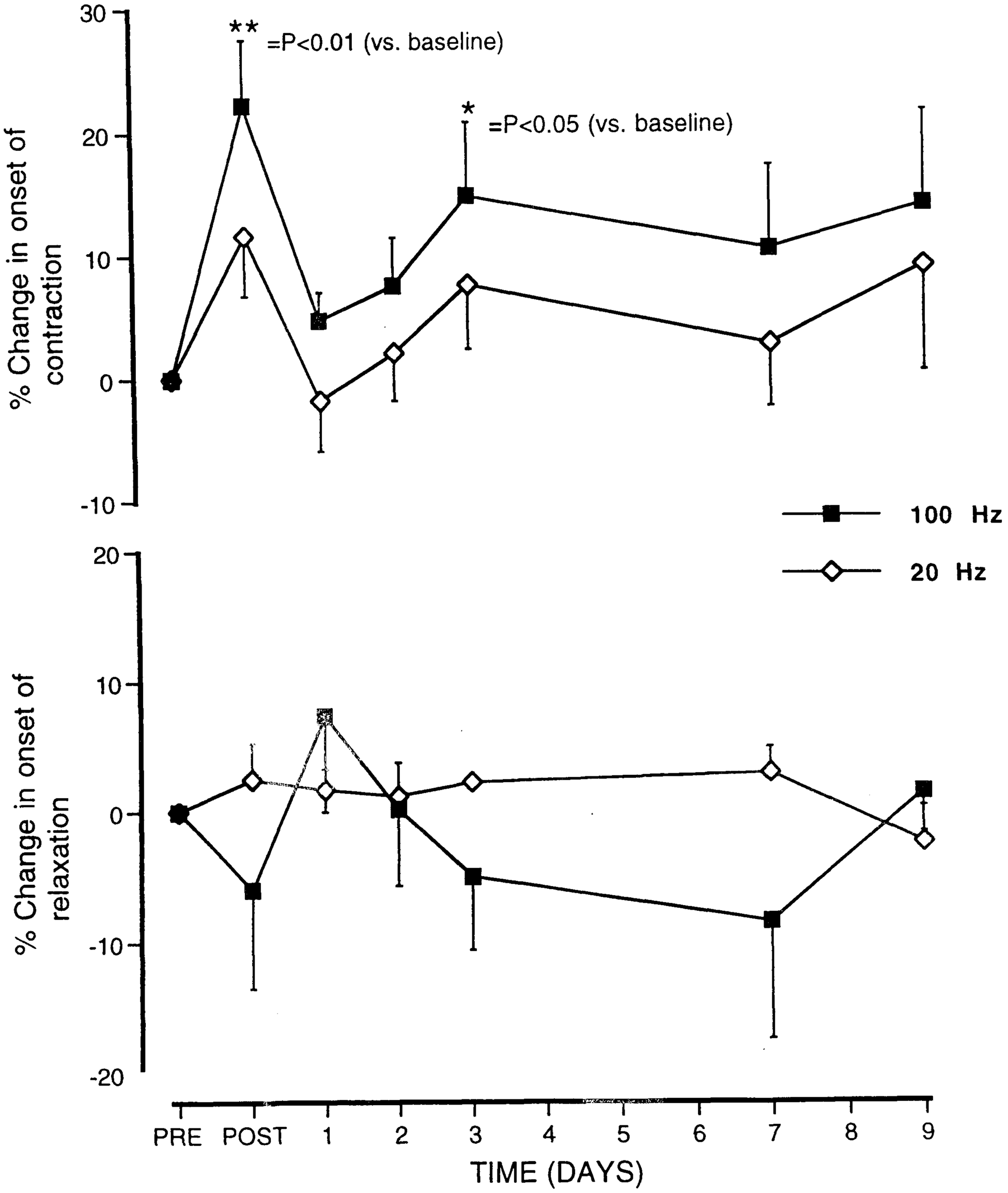


Fig. 3.3. (a and b) Top: % Change from pre-exercise in the time delay for the onset of contraction following a single bout of stimulated eccentric exercise (mean +/- SD), P values using a t-test. Bottom: % Change from pre-exercise in the time delay for the onset of relaxation following a single bout of stimulated eccentric exercise (mean +/- SD).

3.5. Discussion.

In the present study, delayed muscle soreness, muscle contraction force impairment, and elevated serum CK activity have been used as indirect evidence of skeletal muscle damage following eccentric exercise. This study further examined other indices of muscle function, namely contraction and relaxation kinetics, in order to examine the effect of stimulated eccentric exercise on these indices.

The force loss immediately post exercise may reflect the combined effects of muscle fatigue and exercise-induced damage. However, the continued force loss on subsequent days may reflect increasing ultra-structural damage observed by previous authors using eccentric exercise models (e.g. Friden et al, 1983; Jones et al, 1986). A recovery in force during the days after eccentric exercise has been previously reported (Clarkson et al, 1992; Nosaka et al, 1991), although these authors acknowledged that progressive ultra-structural damage may develop during the time that strength was recovering. The force difference between the MVC and the MVS possibly indicated the extent of inhibition of motor unit recruitment during performance of isometric strength tests in the days post exercise. The consistent 10 % increase in force using the MVS technique compared to the MVC, suggested that it was not a motivational problem, or a problem associated with soreness. The MVS contraction was still greater than the MVC contraction even when soreness was back to baseline (e.g. on day 9 post exercise). Since the force increases using the MVS technique were only observed after the exercise bout, this may indicate a central limiting mechanism which may restrict recruitment of partially damaged or vulnerable motor units.

The relative change observed in the time delay associated with the onset of contraction demonstrated a biphasic response (see Fig.3), with an extended delay immediately post exercise and again on day 3 post exercise. The transient nature of this response may reflect the compounding effects of different causes, where the initial increased delay could be due to fatigue, and the delays observed on day 3 may reflect progressive muscle fibre degeneration within a susceptible fibre population (see 1.2.). Although the delay was significant with the 100 Hz stimulus and not with the 20 Hz stimulu (reasons for which are, at present, undetermined) the similarities in the trends described in Fig. 3a may suggest that similar delaying mechanisms were operating with both stimuli.

Immediately post exercise, a fatigue and/or damage induced failure of excitation-contraction coupling may contribute to the extended delay between the application of PES and the onset of muscular contraction. In a fatigued muscle, K^+ accumulation in the T tubules and inter-fibre spaces would increase the excitation threshold of the muscle membrane, and force production would be compromised if accumulation of K^+ interfered with the conduction of the action potential (Jones, 1981). Alternatively, an increased Ca^{2+} concentration in the T tubules may explain the uncoupling of the action potential from contraction during fatigue (Bianchi and Narayan, 1982).

The extended delay in the onset of contraction following an external stimulus 3 days post exercise, may indicate a damage-induced delay in excitation-contraction coupling attributable to secondary degradation processes. This could reflect neuromuscular block, reduced excitability, or defective action potential propagation over the muscle fibre sarcolemma. Failure of the neuromuscular junction to initiate an action potential may reflect a damage-induced disturbance of the metal ion homeostasis in the neuromuscular region, causing a delay in the transmission of the action potential to the post synaptic muscle fibre membrane i.e. a similar scenario to the fatigued state. However, in the present study, the use of percutaneous electrical myostimulation would potentially over-ride the neuromuscular junction, indicating that this was not the site of the increased latency of contraction. Adenosine triphosphate (ATP) dependent K^+ channels in muscle showed an increase in activity at decreased intracellular pH, and Ca^{2+} dependent K^+ channel activity would be promoted by an elevated intracellular Ca^{2+} concentration (Fitts, 1994). K^+ efflux from the myoplasm, attributed to either accelerated ion channel activity or cell membrane leakage, would contribute to resting membrane potential depolarisation and to a reduced action potential amplitude. This may ultimately lead to a depolarisation block of the sarcolemma or T tubular action potential. These mechanisms could potentially increase the temporal offset between stimuli and the onset of contraction.

A more rapid force loss in muscle stimulated at short muscle lengths has been recorded (Sacco et al, 1994), although this could not be explained in terms of neuromuscular junction failure. These authors cited T tubule compression and loss of lumen volume in T tubules as a causative mechanism for the enhanced force loss at short muscle lengths. Swelling and oedema in muscle damaged by eccentric exercise (Friden et al, 1986; Crenshaw et al, 1994) could compress T tubules and present a similar intramuscular environment to that reported by Sacco et al, (1994). However,

the late appearance of swelling following exercise (Clarkson et al, 1992) cannot account for the contraction delays observed immediately post exercise in the present study. Therefore, it is proposed that although oedema within the tissue may contribute to the extended delay observed 3 days post exercise, the delay immediately post exercise cannot be attributable to oedema.

Chronic depression of the 20:100 ratio suggested the presence of low-frequency fatigue (Edwards et al, 1977), and previous work attributed this to a reduced tetanic intracellular Ca^{2+} concentration rather than changes in the extracellular ionic composition (Westerblad et al, 1993). Low-frequency fatigue in single fibres could not be explained in terms of failure of the action potential conductance down the T tubules, myoplasmic buffering, or to changes in SR pumping. Westerblad et al, (1993) proposed that low-frequency fatigue was most likely caused by reduced Ca^{2+} release from the SR, or structural damage to one of the proteins involved in excitation-contraction coupling. However, the same authors expressed caution in extrapolating these mechanisms to human systems.

Distention of the SR post exercise is thought to be transient (Byrd, 1992), which may suggest that ionic disturbance in the T tubule is a more likely explanation of the increased delay in contraction. No significant changes were observed in the time between the onset of contraction and attainment of 70 % of the mean tetanic force produced during a 1s pulse of 100 Hz stimulation, and this may suggest that rapid Ca^{2+} release and uptake from the SR at the 100 Hz frequency were unaffected by this eccentric exercise model. Also relaxation appeared to be unaffected by the stimulated eccentric exercise model, with no significant differences recorded in the time delay between the end of stimulation and the onset of relaxation, or the time to reach 70 % of the total relaxation. Therefore, the SR's affinity for Ca^{2+} appeared unaltered, which may suggest this was not the site of failure in excitation-contraction coupling. However, variability in the relaxation data may account for the lack of any detectable trends in relaxation rates post exercise.

In the present study, a stimulated eccentric exercise model was used to induce temporary damage to human skeletal muscle. The chronic force loss, reduced 20:100 Hz stimulated force ratio, and delayed elevation of serum CK activity, recorded in the present study, are characteristic of eccentric exercise-induced muscle damage. An increased time delay between the start of stimulation and the onset of contraction was observed, without

changes in the times for relaxation. It is suggested that the cause of this increased delay in excitation-contraction coupling originated in a slowing of the action potential propagation prior to Ca^{2+} release from the SR, and not altered SR functioning.

Chapter 4.

Exercise-induced skeletal muscle damage and adaptation following repeated bouts of eccentric muscle contractions.

Parts of this study have been published as conference proceedings, and have been published as a full paper:

Brown S.J., Child R.B., Day S.H., Donnelly A.E. (1995) The role of eccentric exercise duration in experimental skeletal muscle damage in man. Journal of Physiology 489,148P.

Brown S.J., Child R.B., Day S.H., Donnelly A.E. (1996) Rapid adaptation in human skeletal muscle following eccentric exercise. Journal of Muscle Research and Cell Motility 17(1),163.

Brown S.J., Child R.B., Day S.H., Donnelly A.E. (1997) Exercise-induced skeletal muscle damage and adaptation following repeated bouts of eccentric muscle contractions. Journal of Sport Sciences 15, 215-222.

4.1. Abstract.

Repeated bouts of eccentric muscle contractions were used to examine indirect indices of exercise-induced muscle damage and adaptation in human skeletal muscle. Subjects (18 females and 6 males, mean +/- SD age 20 +/- 1.4 years), performed an initial bout of either 10 (n=7), 30 (n=9), or 50 (n=8) maximum voluntary eccentric contractions of the knee extensors, followed by a second bout of 50 contractions 3 weeks later using the same leg. Muscle soreness was elevated after all bouts ($P<0.05$, Wilcoxon test), although initial bouts reduced the soreness associated with the second bout. Force loss and a decline in the 20:100 Hz percutaneous electrical myostimulation force ratio were observed after all exercise bouts ($P<0.01$, repeated measures ANOVA). Serum creatine kinase activity was elevated following initial bouts of 30 and 50 repetitions ($P<0.01$, repeated measures ANOVA), with no increases following 10 repetitions. No increase in serum creatine kinase activity was observed in any group following the second bout ($P>0.05$, repeated measures ANOVA). It is concluded that skeletal muscle adaptation can be brought about by a single bout of relatively few eccentric muscle contractions. Increasing the number of eccentric muscle repetitions did not secure an increased prophylactic effect on skeletal muscle.

4.2. Introduction.

An adaptation has been demonstrated in muscle which has been subjected to eccentric muscle contractions (Newham et al, 1987), such that indirect indices of muscle damage are attenuated following repeated bouts (see 1.5.). This adaptation has been referred to as the "repeated bout effect" by previous authors (Ebbeling and Clarkson, 1990).

Following repeated bouts of downhill running (Byrnes et al, 1985), muscle soreness and serum creatine kinase (CK) activity were reduced when the bout was repeated 3 or 6 weeks later, but not following an 8 week interval. A short duration eccentric exercise bout performed 2 weeks prior to a bout of 70 repetitions (Clarkson and Tremblay, 1988) inferred the adaptation, and early performance of a second bout (Ebbeling and Clarkson, 1990) did not initiate a setback in the recovery process. Speculation on the mechanisms for the adaptation have included damage and removal of a pool of susceptible or vulnerable fibres (see 1.5.1.), increased ability to repair initial damage (Newham et al, 1987; Clarkson and Tremblay, 1988),

and increased muscle connective tissue content (Lapier et al, 1995). It has been speculated (Ebbeling and Clarkson, 1990) that since an elevation of serum CK activity does not generally occur after a repeated bout (Newham et al, 1987), the site of adaptation may be at the muscle membrane and not the contractile proteins.

The duration of eccentric exercise required to induce the prophylactic measure has not previously been determined. Manipulating initial eccentric exercise bout duration, and the subsequent effects of this on muscle adaptation to a repeated bout of eccentric exercise, has not previously been studied in detail. Therefore, using indices of muscle function (see 1.3.2. and 1.3.3.), measurements of muscle soreness and serum CK activity (see 1.3.1. and 1.3.4.), this study aimed to determine the extent to which adaptation to eccentric exercise was dependent on the number of eccentric muscle actions performed in a preceding bout. It was hypothesised that an initial bout of eccentric exercise would induce a protective effect against a second bout, and that an increased exercise duration during the initial bout would further enhance the protective effect.

4.3. Methods.

4.3.1. Subjects.

Twenty four volunteers were randomly assigned to one of three groups. Group 1 (6 female, 1 male; mean +/- SD age 20.5 +/- 1.1 years; mean +/- SD mass 68.3 +/- 9.3 kg) performed 10 repetitions, Group 2 (6 female, 3 male; aged 20.1 +/- 0.8 years; mass 63.6 +/- 13.9 kg) performed 30 repetitions, and Group 3 (6 female, 2 male; aged 22.1 +/- 2.4 years; mass 69.3 +/- 11.5 kg) performed 50 repetitions. All gave informed consent and attended the laboratory for a single familiarisation session. Selected volunteers had refrained from weight training activities for at least 6 months prior to the study, and Ethics Committee approval was obtained for this study.

4.3.2. Exercise Bout.

Volunteers performed two bouts of maximum voluntary knee extensor eccentric muscle contractions, at an angular velocity of 1.05 rad.s^{-1} through a 1.75 rad range of motion. Exercise bouts were performed on a Kin-Com isokinetic dynamometer (Chattecx, Tnn., USA). Subjects were required to

perform a maximum isometric contraction against the dynamometer lever arm for approximately 1 s prior to the initiation of the eccentric contraction. With continued maximum effort, subjects were required to resist the forced lengthening of their knee extensors. Throughout the isometric and eccentric muscle contraction period, subjects were verbally encouraged to maximally contract the knee extensors. The force produced was continuously displayed to the experimenter to indicate the strength of contraction. Each eccentric contraction lasted approximately 1.6 s, with a 10 s recovery period between each contraction. Each subject relaxed the knee extensors at the end of the eccentric contraction, and during the recovery phase the relaxed leg was returned to the starting position by the experimenter. Since angular velocity, range of motion, and recovery period between repetitions were standardised, the number of repetitions performed in each bout was equivalent to the eccentric exercise duration. The second bout was performed 21 days after the first bout and consisted of 50 repetitions using the same leg as that exercised in the first bout. Body position, approximate centre of rotation of the knee joint, and dynamometer lever arm length were consistent between bouts for each subject.

4.3.3. Force Data Collection.

Measures of muscle function were obtained from each subject using previously described methods (see 2.2.2.). These measures of muscle function were collected before, immediately after, and on days 1, 2, 3, 7, and 9, following each bout. These time points were chosen in an attempt to identify any acute changes within the initial 72 hours, and a more long term response occurring one week after the bout. Measures were collected in duplicate and in a randomised order, and consisted of :

1. knee extension maximum isometric voluntary contraction force at a knee flexion angle of approximately 1.57 ad. (MVC),
2. the mean tetanic force produced during a 1 s pulse of 100 Hz percutaneous electrical myostimulation (PES),
3. the mean tetanic force produced during a 1 s pulse of 20 Hz PES.

Measures 2 and 3 were used to calculate the 20:100 Hz force ratio, and this ratio was used as an index of low-frequency fatigue (see 1.3.3.). PES was applied to the knee extensor muscles using previously described techniques (see 2.2.2.).

4.3.4. Other indices of muscle damage.

Delayed onset muscle soreness (DOMS) was assessed daily by a questionnaire (see 2.1.).

A 10 ml venous blood sample was collected from the antecubital fossa (see 2.3.) pre-exercise, and on days 1,2,3,7 and 9 following the bout. Blood samples were allowed to clot at room temperature for 30 min prior to centrifugation at 1500 g for 10 min, thus allowing the serum layer to be recovered. Serum samples were stored at -20 degrees C prior to measurement (in duplicate) of creatine kinase (CK) activity (see 2.4.).

4.3.5. Statistical analysis.

Parametric data were analysed using repeated measures analysis of variance (ANOVA) with 2 within factors (Bout, i.e. the difference between the 2 periods of the study, and Time, i.e. the changes over time after each bout) and 1 between factor (Group). Due to the relatively small numbers in groups and the unequal numbers in each group, parametric data were treated in the following ways:

1. MVC data were expressed as % changes to normalise the values and reduce inter-individual variability;
2. Force loss at low frequency of stimulation (20 Hz) was expressed as a ratio of 100 Hz tetanic force;
3. Serum CK activities were subjected to logarithmic transformation. This meant that positively skewed distributions tended toward symmetry, and since relatively large mean CK values have a large SD and smaller mean CK values have a smaller SD, logarithmic transformation reduced the SD of the values with a high activity more than it reduced the SD of the values with lower activity (Howell, 1992).

Soreness data were analysed using a Wilcoxon test for matched pairs.

The prophylactic effect of a single eccentric exercise bout was determined by comparing the response of the first bout in group 3 to the response of each group following the second bout using ANOVA with post hoc non-paired t tests for respective data points. For the post hoc analysis, significance levels were adjusted using a Bonferroni t correction such that $P<0.025$ was the minimum level of accepted significance. The Bonferroni t corrected P value was based on the comparison of groups 1 and 2, bout 2, against group 3 bout 1 (group 3 bout 2 was compared to group 3 bout 1

using the initial ANOVA). The adjusted P value was determined by alpha (0.05) divided by the number of repeated comparisons (2), such that $P<0.025$ was the minimum level of accepted significance. It was assumed that a typical response to 50 maximum voluntary eccentric actions was observed in group 3 following the first bout, thereby serving as a control for all the groups when performing the second bout.

4.4. Results.

Eccentric muscle forces produced by each group during the second exercise bout were recorded on the dynamometer. All groups showed a similar overall decline in mean eccentric muscle force during the second bout. Mean +/- SD force on repetition 1 was 770 +/- 75 N, 758 +/- 96 N, and 717 +/- 49 N for groups 1, 2, and 3 respectively. Mean +/- SD force on repetition 50 was 588 +/- 88 N, 559 +/- 74 N, and 495 +/- 53 N for groups 1, 2, and 3 respectively. The consistency of the force decline in the 3 groups suggested that previous bout duration did not affect performance during the second bout. Muscle soreness (DOMS) data are presented in Table 4.1. Soreness was elevated above baseline for 3 days after all bouts regardless of duration ($P<0.05$, Wilcoxon test). Groups 2 and 3 continued to experience DOMS up to 4 days and 6 days post-exercise respectively. Group 3 recorded higher soreness than groups 1 and 2 on days 5 and 6 ($P<0.05$, Wilcoxon test). DOMS was evident following the second bout for all groups, remaining significantly elevated above baseline for 2 days post-exercise in group 1, and 3 days post-exercise in groups 2 and 3. Less soreness was recorded in groups 1, 2 and 3 following bout 2 on days 2 (group 1 only), 3, 4, 5, and 6 (all groups) compared to the corresponding day post-exercise in group 3 following bout 1.

Normalised MVC data are presented in Fig. 4.1. Mean +/- SD bout 1 pre-exercise MVC values were 424 +/- 145 N, 421 +/- 190 N, and 393 +/- 124 N for groups 1, 2, and 3 respectively. Mean +/-SD bout 2 pre-exercise MVC values were 427 +/- 110 N, 500 +/- 305 N, and 370 +/- 166 N for groups 1, 2, and 3 respectively. Following the first bout, group 3 %MVC on days 3, 7, and 9 post-exercise were significantly lower than the corresponding data points for groups 1 and 2. Following the second bout, groups 1 and 3 %MVC were higher than the corresponding days post-exercise in group 3 following bout 1. The 20:100 Hz force ratio data are presented in Fig. 4.2. With the exception of group 1, all ratios showed a trend toward recovery on the days subsequent to the first bout, and this trend was repeated following the

second bout. No differences were recorded between groups following bout 1, although following the second bout, post-exercise values for groups 1 and 3 were significantly greater than the corresponding value for group 3 following bout 1. A significantly higher 20:100 Hz force ratio was recorded on day 9 post-exercise in group 3 following the second exercise bout.

Serum CK activities for all subjects were within the normal range before the study (12-106 $IU.l^{-1}$). Due to the large intra-subject variability with CK measures throughout the time course of the normal recovery period following the initial insult, values were expressed in logarithmic terms (shown in Fig.3). Differences in the serum CK activity of males and females have been previously reported although in the present study this difference was not observed. Removal of the males' serum CK activity values from the analysis produced the same trends with the same statistical significance; therefore, the data presented are for the groups outlined in methods. Following bout 1, peak serum CK activity was recorded on day 1 for group 1, day 2 for group 2, and day 3 for group 3. Serum CK activity remained significantly elevated above baseline for 7 days post-exercise in group 3. No significant differences were recorded in serum CK activity following the second bout in all groups. Serum CK activities on days 2, 3, and 7 following the second bout in all groups was significantly lower than that recorded for the same days following bout 1.

Table 4.1. Median (minimum-maximum) soreness of the knee extensors following two bouts of maximum voluntary eccentric exercise. The number of repetitions varied between groups during bout 1, and all groups performed 50 repetitions during bout bout 2. Values are arbitrary units with statistical analysis using a Wilcoxon test.

	BOUT 1									BOUT 2								
	pre-exercise	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8	pre-exercise	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
Group 1 (10 reps)	6 (6-6)	12[a] (7-14)	15[a] (11-16)	9[a] (6-12)	6 (6-9)	6 (6-6)	6 (6-6)	6 (6-6)	6 (6-6)	6 (6-6)	11[a] (6-21)	14[a,c] (6-23)	10[c] (6-17)	6[c] (6-14)	6[c] (6-9)	6[c] (6-7)	6 (6-6)	6 (6-6)
Group 2 (30 reps)	6 (6-6)	14[a] (8-37)	17[b] (13-24)	10[a] (6-16)	8[a] (6-11)	6 (6-7)	6 (6-6)	6 (6-6)	6 (6-6)	6 (6-6)	13[a] (6-28)	15[a] (6-20)	10[a,c] (6-20)	6[c] (6-16)	6[c] (6-12)	6[c] (6-8)	6 (6-6)	6 (6-6)
Group 3 (50 reps)	6 (6-6)	22[a] (10-27)	20[a] (11-40)	18[a] (8-36)	15[a] (6-25)	12[a] (6-19)	9[a] (6-14)	6 (6-9)	6 (6-6)	6 (6-6)	14[a] (8-21)	17[a] (11-23)	12[a,c] (10-15)	7[c] (6-15)	6[c] (6-13)	6[c] (6-6)	6 (6-6)	6 (6-6)

[a] = P<0.05 vs baseline
[b] = P<0.01 vs baseline
[c] = P<0.05 vs same day, group 3 bout 1

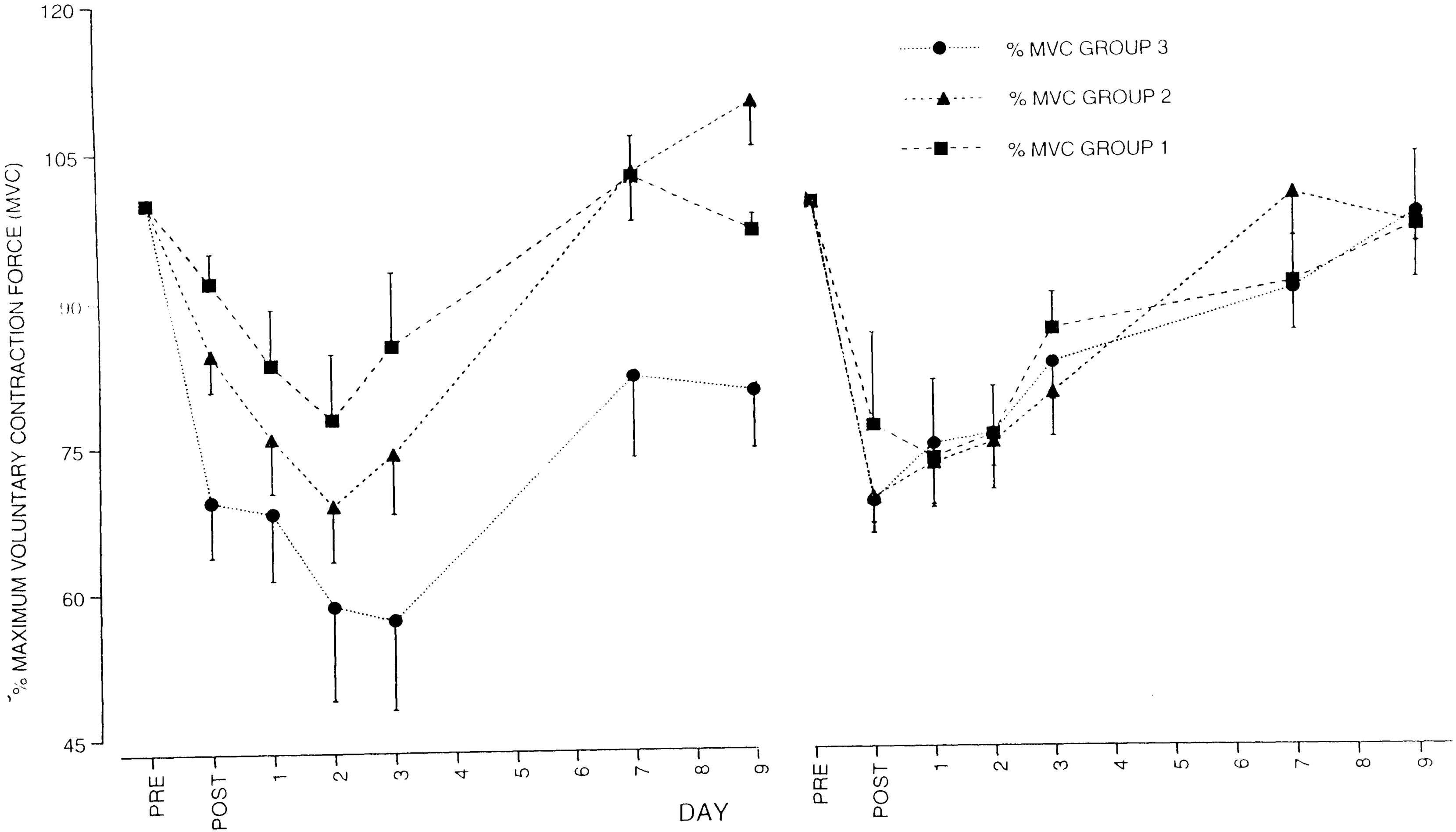


Fig. 4.1. Normalised maximum voluntary contraction force of the knee extensors following 2 bouts of voluntary eccentric exercise. The number of repetitions varied between groups during bout 1, and all groups performed 50 repetitions during bout 2 (Means +/- SD). All groups showed significant changes over time for each bout ($P<0.001$, ANOVA). Groups 2 and 3 showed a significantly different response between bouts ($P<0.05$, ANOVA). Data were normalised for each bout, where pre-exercise values were 100 %.

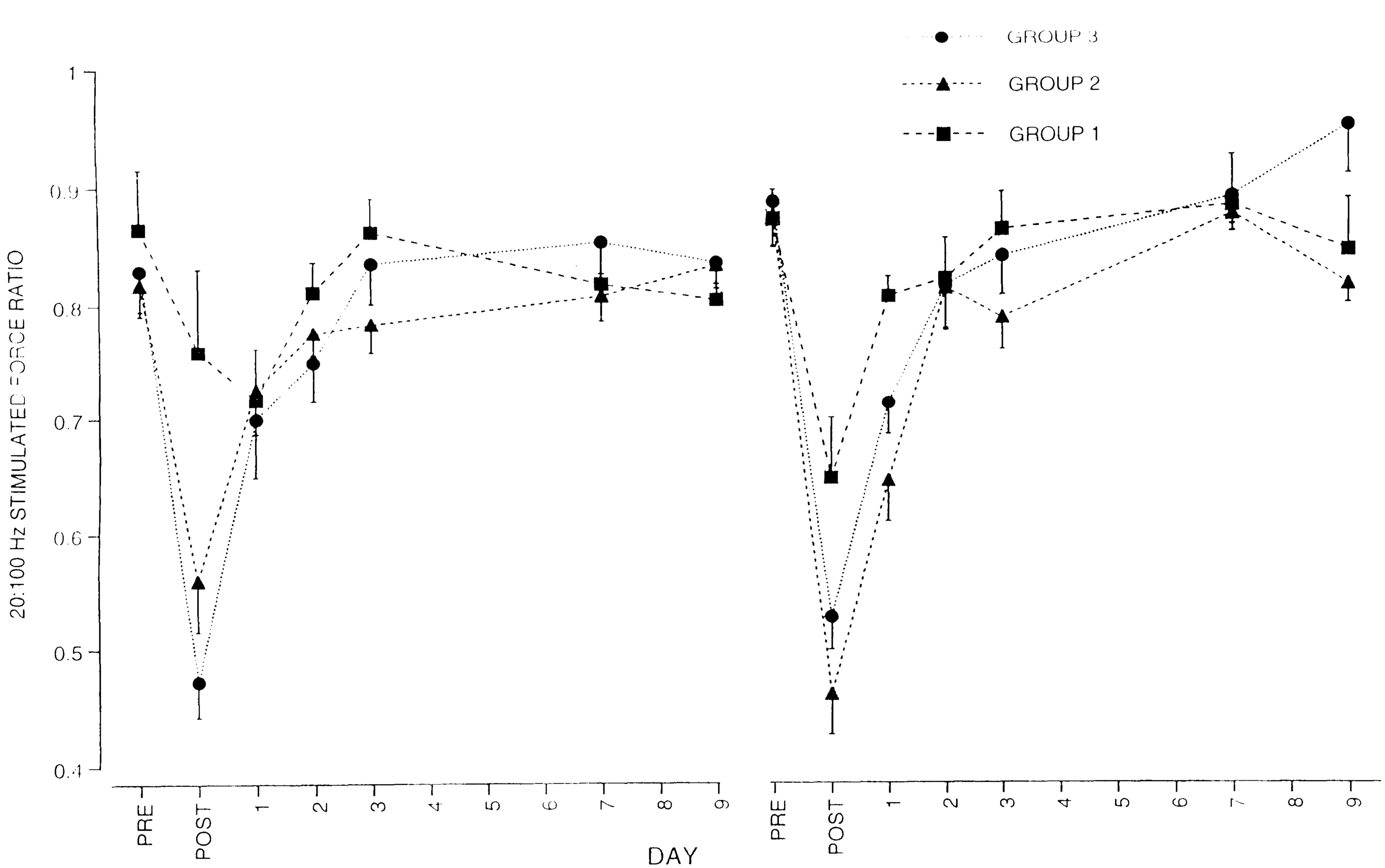


Fig. 4.2. 20:100 Hz stimulated force ratio of the knee extensors following 2 bouts of voluntary eccentric exercise. The number of repetitions varied between groups during bout 1, and all groups performed 50 repetitions during bout 2 (means +/- SD). All groups showed significant changes over time for each bout ($P<0.001$, ANOVA). Group 1 showed a significantly different response between bouts ($P<0.01$, ANOVA).

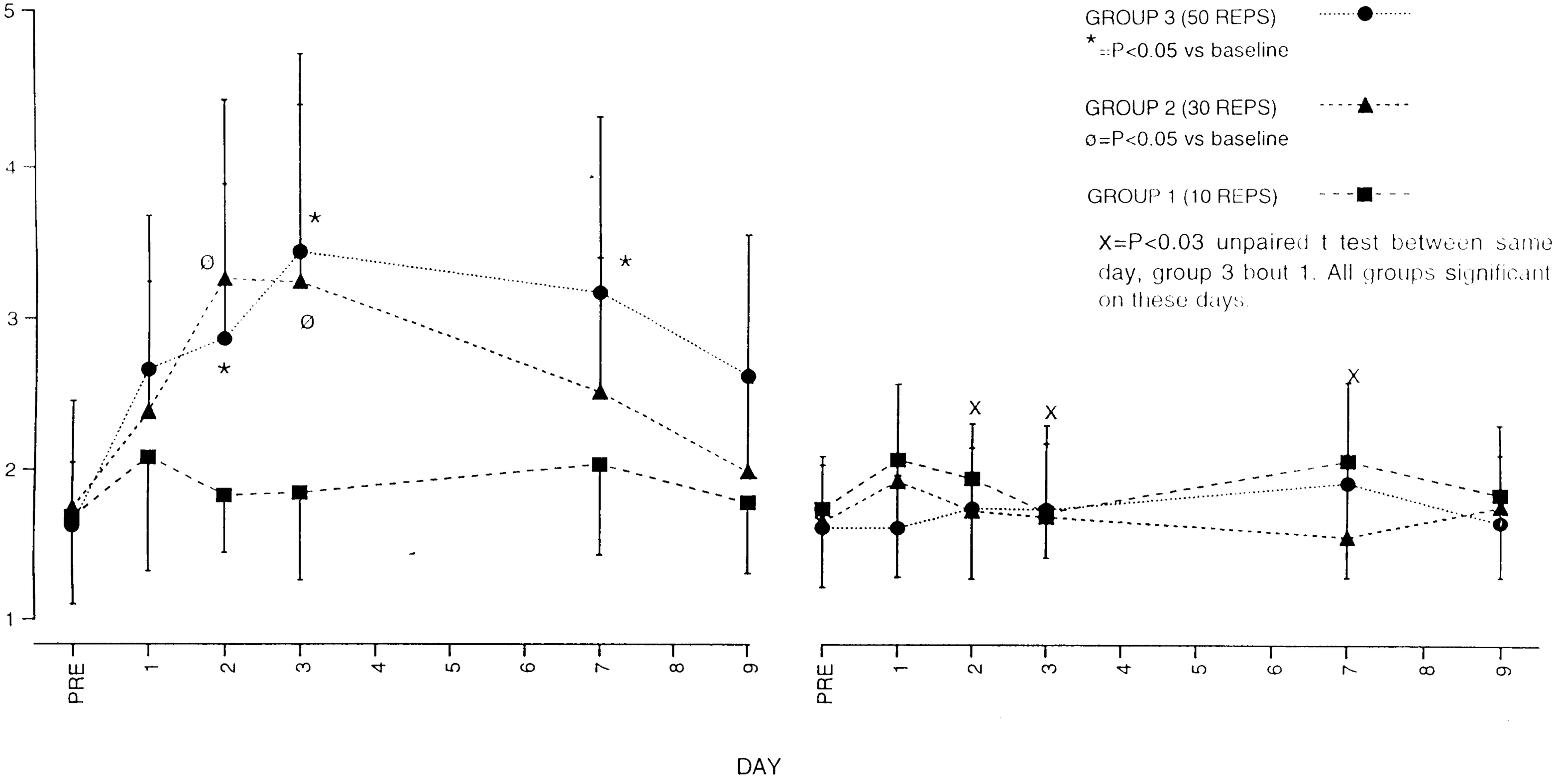


Fig. 4.3. Mean +/- SD changes in log(serum CK activity) with two bouts of voluntary eccentric exercise of the knee extensors. the number of repetitions varied during the first bout (see legend) and all groups performed 50 repetitions during bout 2.

4.5. Discussion.

The present study used indirect indices as evidence of skeletal muscle damage (e.g. soreness, force loss, low-frequency fatigue, serum enzyme activity) following bouts of eccentric exercise in which the number of repetitions were manipulated. Since angular velocity and range of motion were standardised, the repetition number was directly proportional to eccentric exercise bout duration.

4.5.1. Exercise bout duration.

This study attempted to determine the extent to which exercise-induced skeletal muscle damage was dependent on eccentric exercise bout duration. Regardless of initial bout duration, all subjects reported DOMS following the first exercise bout. This may suggest that a common stimulus may trigger the onset of DOMS, possibly a metabolic stimulus such as the localised elevation of the K^+ concentration (Hayward et al, 1991). This may activate group 3 and 4 muscle sensory afferents, thus leading to the sensation of muscle pain (Edwards, 1988). Type 3 sensory afferents are also sensitive to non-noxious mechanical stimuli (Mense and Meyer, 1985), and the involuntary muscle shortening reported following some eccentric exercise protocols (Clarkson et al, 1992) may be further adding to the sensation of muscle pain. Alternatively, the presence of connective tissue breakdown products 24-48 hours after the bout may also contribute to the sensation of DOMS (Abraham, 1977). Since DOMS persisted for longer following exercise bouts of longer duration, this may suggest that other mechanisms are contributing to DOMS in groups 2 and 3. For example, delayed oedema (Jones et al, 1987) and elevated intramuscular fluid pressure (Crenshaw et al. 1995) may be contributing to DOMS, as the temporal characteristics of these phenomena are similar. It has been proposed that secondary muscle fibre degeneration (see 1.2.) following eccentric exercise may be attributable to an inflammatory response (see 1.2.3.). Thus, the continued elevation of DOMS in groups 2 and 3 may be attributable to a localised accumulation of inflammatory mediators (e.g. bradykinin, prostaglandins, and histamine), which may sensitise the muscle sensory afferents (Hayward et al, 1991).

All initial bouts induced a temporary impairment of muscle function, and this appeared to be proportional to the exercise bout duration. It has been suggested that Ca^{2+} dependent proteases and lysosomal acid hydrolases (see 1.2.1. and 1.2.2.) breakdown intramuscular proteins

following eccentric muscle contractions, and therefore the force loss observed in the present study, may reflect enzyme-induced damage to the contractile proteins. If these Ca^{2+} sensitive processes are activated, they appeared to be duration dependent, although the underlying mechanisms are not clear. It has been suggested that high muscle forces during the initial repetitions of an eccentric exercise bout cause the subsequent injury (Child et al, 1995), although this would suggest that all groups in the present study would be equally susceptible to damage following the first bout. However, McCully and Faulkner (1986) reported that evidence of mouse muscle damage (both histological and force loss) increased with an increased duration, although extending the duration beyond 5 min did not result in additional injury. Although speculative, it is possible that the apparent relationship between eccentric exercise duration and force loss may be a result of eccentric contraction within a fatiguing muscle. If muscle fibres which are active during the initial repetitions become fatigued, continuing eccentric muscle contractions may predispose those fibres to some mechanical injury e.g. 'inter-fibre' shear stress. Damage due to a tension imbalance between adjacent sarcomeres has been proposed (Friden and Lieber, 1992), although a similar mechanism acting between active and inactive (possibly due to fatigue or damage) muscle fibres remains to be established.

In the present study, initial bouts of 30 and 50 repetitions caused a significant elevation of serum CK activity in the days following exercise, whereas a short duration bout of 10 repetitions did not significantly elevate serum CK activity. An increased serum CK activity has previously been used as an indirect marker of changes in muscle membrane permeability (see 1.3.4.), and as evidence of injury to muscle fibres (Friden et al, 1989). This may suggest that 10 repetitions caused no changes in muscle membrane permeability, and that both 30 and 50 repetitions were sufficient to induce changes in membrane permeability. However, the relationship between increases in the systemic activities of muscle specific enzymes and muscle damage has been questioned (Volfinger et al, 1994). The results of the present study suggest that a "duration threshold" may exist, above which any initial mechanical damage (see 1.1.) may lead to secondary fibre degeneration (see 1.2.), as evidenced by an increased serum CK activity. Although speculative, it may be that the initial bouts of 30 and 50 repetitions were above this threshold (hence a significant increase in serum CK activity), whereas the 10 repetitions did not induce damage sufficient to

cause secondary degeneration and a concomitant increase in serum CK activity.

4.5.2. The repeated bout effect.

This study also demonstrated that prior eccentric exercise bout duration has little or no influence on the responses of indices of muscle function (20:100 Hz stimulated force ratio and MVC) following a second bout, and only a limited influence on muscle soreness following the second bout. These results suggest that the extent of adaptation is not dependent on prior exercise duration. Thus, no "increased" level of protection is offered to muscle which has performed more repetitions. Previous authors (e.g. Nosaka et al, 1991) have suggested that the greater the "overload" the greater the resulting adaptation, but this suggestion is not supported by the present study. Clarkson and Tremblay (1988) reported a similar experiment to the present study, in which the elbow flexors were exercised initially for a short duration, followed by a second exercise bout of longer duration two weeks later. Essentially, the main difference between the present study and that reported by Clarkson and Tremblay (1988) is that in the present study, the number of repetitions (and therefore the exercise duration) performed in the first bout was varied among groups. Clarkson and Tremblay (1988) reported "faster repair" of damage following a second bout (based on measurements of MVC) when compared to recovery rates following an initial short duration bout. The present study has not supported this finding.

All initial bouts inferred a prophylactic effect against chronic force loss following the second bout, and this effect appeared to be independent of the number of repetitions performed in the initial bout. Data on eccentric contraction forces produced during a second exercise bout have previously been reported (Nosaka et al, 1991), although not under conditions where the initial bout duration was varied. Inter-group consistency in the force decline during performance of the second bout indicated that any adaptation attributable to the first bout did not protect the muscle during the second bout. Whether the declines in eccentric muscle force during the second exercise bout are attributable to fatigue and/or damage, all groups appeared equally susceptible.

For all groups, any increases in serum CK activities following the second bout were not significant. It has previously been reported (Newham et al, 1987) that performance of a single bout of eccentric muscle contractions can substantially attenuate increases in serum CK activity following subsequent eccentric exercise, although the mechanisms of this

adaptation are not known. However, the present study has further demonstrated that increases in serum CK activity in the first bout are not necessarily required in order to attenuate any increases associated with a second bout. Force loss and release of intramuscular constituents following eccentric exercise may represent the removal of irreparably damaged fibres that may have been particularly susceptible to damage (see 1.5.1.). In the present study, the first exercise bout may have induced degeneration within a susceptible myofibre population. However, force loss was observed after the first bout in all groups but only groups 2 and 3 demonstrated an increased serum CK activity. This may suggest that force loss and increased serum CK activity are unrelated in as much as they are phenomena attributable to different mechanisms. It is possible that a short duration bout of 10 repetitions was not sufficient to irreversibly damage a susceptible fibre population, indicated by the lack of an increase in serum CK activity following this bout. Although speculative, this may suggest that part of the mechanism of skeletal muscle adaptation is associated with an improved ability to repair initial micro-injury, and not the removal of vulnerable fibres from the motor unit population. Muscle damage without an associated CK efflux has been proposed (Stauber, 1989; Kolumainen et al, 1995), and impairment of muscle function can be present without accompanying evidence of muscle damage (Balnave and Thompson, 1993). These observations are supported by the present study.

Alleviation of increased CK activity following eccentric exercise by an initial bout that appeared to cause little or no damage has been demonstrated. This study has presented evidence suggesting that adaptation is not necessarily dependent on previous damage during which susceptible fibres are removed from the motor unit population. It is speculated that since damage appears not to be a prerequisite for adaptation, part of the mechanism of adaptation may be linked to an improved ability to repair the initial micro-injury. This study has also demonstrated that an increased number of eccentric muscle contractions performed in an initial bout does not secure an increased prophylactic effect against subsequent, potentially damaging, eccentric exercise bouts.

Chapter 5.

Indices of human skeletal muscle damage and collagen breakdown following concentric and eccentric exercise.

Parts of this study have been accepted for a conference:

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A part of the material in this chapter, namely force data, were collected by S.H. Day, and are reported with permission.

5.1 Abstract.

Skeletal muscle function, and indirect biochemical markers of muscle damage and connective tissue breakdown were recorded for up to 9 days following concentric and eccentric exercise in 9 untrained subjects (mean +/- SD age 26 +/- 2 years). Bouts were performed on an isokinetic dynamometer using the knee extensors of a single leg, and consisted of 50 maximal effort contractions. Bouts were separated by 21 days. The concentric exercise bout induced no changes in knee extensor maximum voluntary isometric contraction force (MVC), or induced any changes in the serum enzyme activities of creatine kinase (CK), lactate dehydrogenase (LDH-1), or alkaline phosphatase (ALP). Concentric exercise induced no changes in markers of connective tissue breakdown, namely plasma hydroxyproline (HP) and serum type 1 collagen concentration. Eccentric contractions induced a 23.5 +/- 19.0 % decrease in MVC post exercise ($P<0.05$, Duncan post hoc following repeated measures analysis of variance(ANOVA)), and increased the activities of CK and LDH-1 in serum by 430 +/-792 $IU.l^{-1}$ and 5 +/- 11 $IU.l^{-1}$ respectively on day 3 post exercise, and by 133 +/- 159 $IU.l^{-1}$ and 11 +/- 13 $IU.l^{-1}$ respectively on day 7 post exercise. Eccentric exercise induced no significant changes in plasma HP, but increased the serum concentration of type 1 collagen on days 1 and 9 post exercise ($P<0.05$, Duncan post hoc following repeated measures ANOVA).

It is concluded that eccentric and not concentric exercise may result in temporary muscle damage, and that collagen breakdown may also be affected by eccentric exercise. With caution, the use of indirect markers of collagen breakdown may be used to identify exercise-induced damage to connective tissue structures, although plasma HP and serum type 1 collagen appear to respond differently to eccentric exercise.

5.2. Introduction.

The susceptibility of muscle connective tissue to injury during eccentric muscle contractions may be increased due to the high forces encountered (see 1.4.). Thus, analysing the circulating levels of a specific collagen sub-type found extensively in tendon, epimysium, and to a lesser extent, perimysium, e.g. type 1 collagen (Duance et al, 1977), and/or products of collagen breakdown e.g. collagen imino acids such as hydroxyproline (HP), may indicate exercise-induced damage to connective

tissue. However, to date, the balance between collagen synthesis and degradation following exercise has not been fully determined.

Serum levels of type 1 collagen have been shown to be elevated 3 days following eccentric muscle contractions but not following concentric exercise (Saxton and Donnelly, 1994). Serum levels of the carboxyterminal telopeptide region of type 1 collagen, also an index of collagen breakdown, did not increase at either 5 min or 1 hour following short term high intensity exercise (Kristofferson et al, 1995). Elevated levels of plasma hydroxyproline (HP) have been used to indicate exercise-induced injury to connective tissue (Murguia et al, 1988), although Virtanen et al (1993) reported no changes in serum HP following high intensity concentric exercise.

Circulating levels of collagen propeptides, which are released into the circulation following cleavage from procollagen during new collagen synthesis, have shown a variable response to exercise. Serum concentrations of the carboxyterminal propeptide of type 1 procollagen decreased 1 hour following high intensity concentric exercise, but started to increase 2 days after the exercise (Virtanen et al, 1993). These authors suggested that the transient decrease in the production rate of type 1 collagen could be attributed to strain on the musculoskeletal system. Short term high intensity cycling exercise did not affect serum concentrations of the carboxyterminal propeptide of type 1 procollagen (Kristofferson et al, 1995) for up to 1 hour post exercise, however, these authors acknowledged that the short time span may not have been suitable for studies of acute exercise on collagen metabolism. Serum concentrations of the aminoterminal propeptide of type 3 procollagen were increased 2 days after the termination of a 24 hour cross-country run (Takala et al, 1986), but not following a 24 hour cross-country skiing event (Takala et al, 1986) or following high intensity concentric exercise (Virtanen et al, 1993). Increased type 3 collagen deposition has been shown to occur during the regeneration of exercise-induced muscle injury (Myllyla et al, 1986). Therefore, the eccentric component of the running protocol may have caused some delayed muscle damage which increased type 3 collagen deposition during the repair/regeneration process.

The present study investigated the effects of concentric and eccentric exercise on indirect indices of muscle damage and collagen breakdown in untrained human subjects. It was hypothesised that concentric exercise would not induce muscle damage or collagen breakdown, whereas the bout of eccentric exercise would induce muscle damage and increase collagen breakdown.

5.3. Methods.

5.3.1. Subjects.

Nine subjects, 4 male 5 female, mean (+/-SD) age 26 +/- 2 years, all of whom had not participated in resistance training for more than 6 months previously, signed informed consent documents and attended the laboratory for a single familiarisation session. The University of Wolverhampton Ethics committee approved this study.

5.3.2. Exercise Bout.

Two bouts of isokinetic exercise (at 1.05 rad.s^{-1}) were performed on a Kin-Com isokinetic dynamometer (Chattecx, Tnn., USA). All subjects performed an initial bout of 50 maximum voluntary concentric contractions of the knee extensors of a randomly chosen leg. The exercise bout was performed by each subject lying in a prone position, thus enabling a range of motion at the knee from near full flexion to full extension. Each subject performed a maximum isometric contraction against the dynamometer lever arm for approximately 1 s prior to the initiation of the concentric contraction. With continued effort, subjects extended the knee through the range of motion (1.75 rad.), thus performing a concentric muscle contraction. Subjects relaxed their knee extensors at the end of the concentric contraction, and during the recovery phase, the relaxed leg was returned to the starting position by the experimenter.

Three weeks later, all subjects performed a second exercise bout consisting of 50 maximum voluntary eccentric contractions of the knee extensors of the same leg. For this second bout, all exercise conditions were the same as the initial concentric bout, except subjects were required to maximally resist the forced lengthening of their knee extensors, thus performing an eccentric contraction. During the concentric and eccentric muscle contractions, force was continually displayed and verbal encouragement was given throughout the bouts. Each contraction lasted approximately 1.6 s and each contraction was separated by a 10 s recovery period.

5.3.3. Indirect indices of skeletal muscle damage.

Duplicate measures of knee extensor maximum isometric voluntary contraction force at a knee flexion angle of approximately 1.57 rad (MVC) were collected in a randomised order, before and after exercise, and on days 1, 2, 3, 7, and 9, following each bout (see 2.2.). Delayed onset muscle soreness (DOMS) was assessed daily by a questionnaire incorporating 6 sites on the anterior muscles of the leg. Details of this questionnaire and method of soreness assessment have been previously described (see 2.1.).

A 10 ml venous blood sample was collected from the antecubital fossa (see 2.3.) before exercise, and on days 1, 2, 3, 7, and 9, following each bout. For serum, 5 ml blood samples were allowed to clot at room temperature for 30 min prior to centrifugation at 1500 g for 10 min (Megafuge 1.0, Heraeus Sepatech, Germany), thus allowing the serum layer to be recovered. For plasma, 5 ml blood samples were collected into tubes containing ethylenediamine-tetracetic acid (EDTA), mixed, and centrifuged at 1500 g for 10 min, thus allowing the plasma layer to be recovered. Samples were stored at -20 degrees C prior to analysis. Serum creatine kinase activity (CK) was measured using an enzymatic kit (see 2.4.), and serum lactate dehydrogenase-1 activity (LDH-1) was measured using an enzymatic kit (see 2.5.).

5.3.4. Indirect indices of connective tissue turnover.

Serum alkaline phosphatase activity (ALP) was measured using an assay kit (see 2.6.). Serum type 1 collagen concentration was measured using an inhibition enzyme linked immunosorbant assay (ELISA) (see 2.9.), and plasma HP was determined using a method previously described (see 2.8.3.). The method for HP determination included a step in which proteins, including non-collagenous protein bound HP and other interfering substances, were precipitated out. Subsequent sample hydrolysis released peptide-bound HP, therefore this assay measured both free and bound HP in plasma.

5.3.5. Statistical analysis.

Soreness data were analysed using Wilcoxon signed rank tests. Parametric data were analysed using repeated measures analysis of

variance (ANOVA) with post hoc analysis using a Duncan multiple range test. If the overall difference between bouts was significant with the ANOVA, a post hoc comparison between bouts for the same time points was carried out using a Student t-test for paired data. Data for serum CK activity were analysed following logarithmic transformation (see 4.3.5.). Values reported are means +/- SD, and the accepted level of significance was $P<0.05$.

5.4. Results.

No soreness was reported following the concentric exercise bout, although following the eccentric bout, DOMS was significantly above pre-exercise values on day 1 ($P<0.01$, Wilcoxon test), and up to day 3 post exercise (day 2 and day 3 vs. pre-exercise, both $P<0.05$, Wilcoxon test).

Mean +/- SD pre-exercise knee extensor MVC values were 371 +/- 127 N and 395 +/- 126 N for concentric and eccentric exercise bouts respectively, and these were not significantly different ($P>0.05$, t test). No significant changes in the knee extensor MVC were recorded following the concentric exercise bout (data normalised to % change are shown in Fig. 5.1.), although a significant decrease in MVC was recorded following eccentric exercise (pre-exercise vs. post exercise, $P<0.05$, Duncan post hoc following repeated measures ANOVA). The overall difference between bouts was significant ($P<0.05$, repeated measures ANOVA), however, there were no significant differences between paired data points.

Mean +/- SD serum enzyme activities following the concentric and eccentric exercise bouts are shown in table 5.1. Pre-exercise enzyme activities were within the normal range for both exercise bouts, and returned to values within the normal range by day 9 post exercise following both bouts. No changes in CK, LDH-1, and ALP were recorded following the concentric exercise bout. Following the eccentric exercise bout, significant increases in CK and LDH-1 were recorded on days 3 and 7 post exercise ($P<0.05$, Duncan post hoc following repeated measures ANOVA), whereas no increases in serum ALP activity were recorded following eccentric exercise.

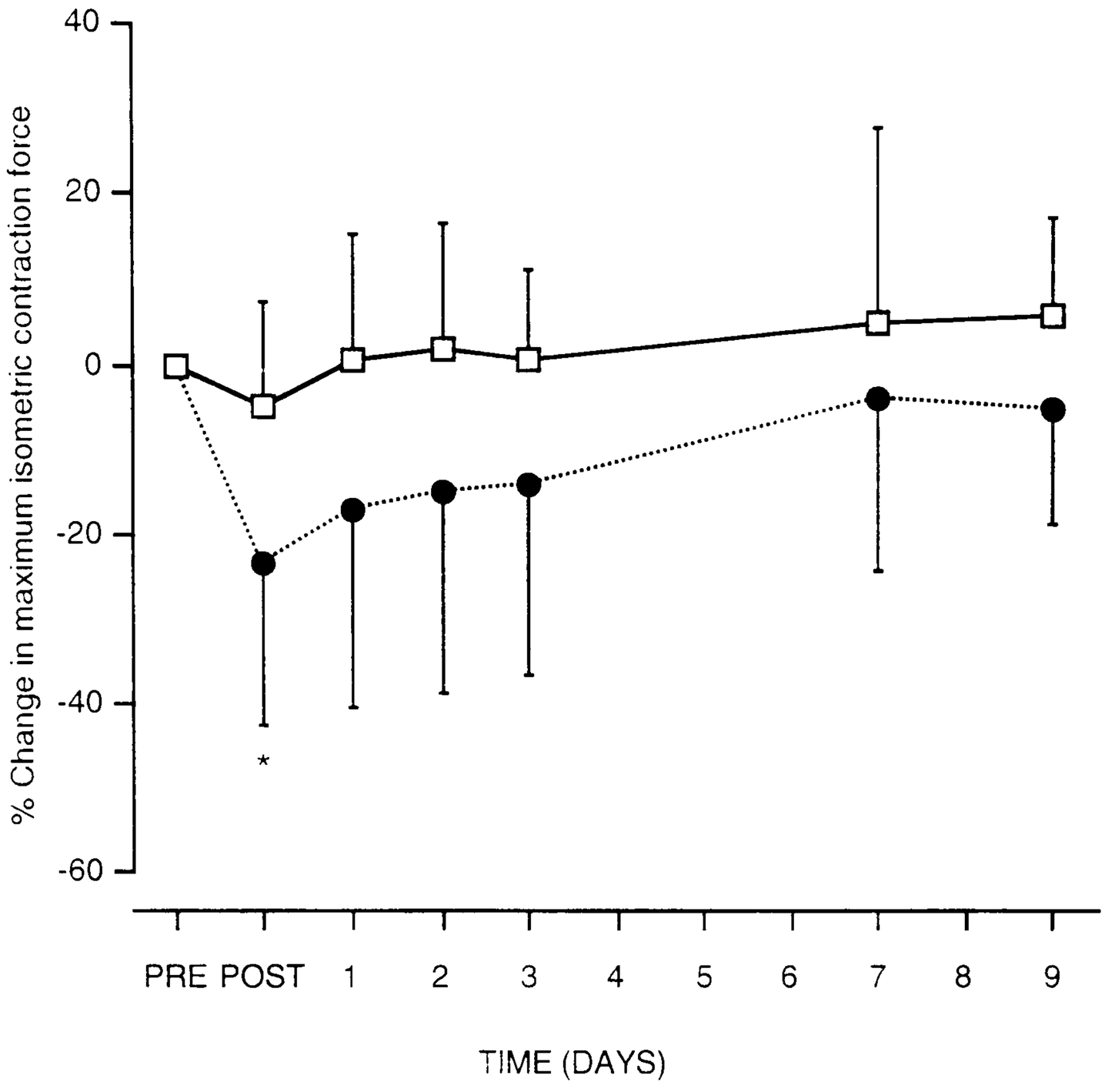

Fig.5.1. % change in maximum isometric contraction force of the knee extensors following concentric exercise (open squares) and eccentric exercise (closed circles). Values are means +/- SD, and P values vs.PRE using Duncans post hoc following repeated measures ANOVA. (*=P<0.05).

		PRE	DAY 1	DAY 2	DAY 3	DAY 7	DAY 9
CON.	CK	44 (16)	61 (29)	51 (26)	44 (25)	64 (66)	54 (30)
	LDH-1	81 (12)	81 (10)	78 (10)	81 (9)	87 (12)	82 (17)
	AL	101 (29)	102 (31)	100 (29)	101 (30)	105 (26)	102 (31)
ECC.	CK	56 (23)	79 (48)	78 (30)	486 (792)a	189 (159)ac	66 (22)
	LDH-1	85 (10)	81 (8)	84 (13)	90 (11)b,c	96 (13)b,c	89 (5)
	ALP	94 (17)	98 (30)	104 (26)	98 (32)	104 (24)	108 (34)

Table 5.1. Serum enzyme activities following concentric and eccentric exercise of the knee extensors of a single leg. CK= creatine kinase, LDH-1=lactate dehydrogenase isoenzyme 1, ALP= alkaline phosphatase. Values are means +/- SD. a= P<0.05 vs pre-exercise on log. values using Duncan post hoc following repeated measures ANOVA. b= P<0.05 vs pre-exercise values using Duncan post hoc following repeated measures ANOVA. c= P<0.05 between bouts using a paired Students t test.

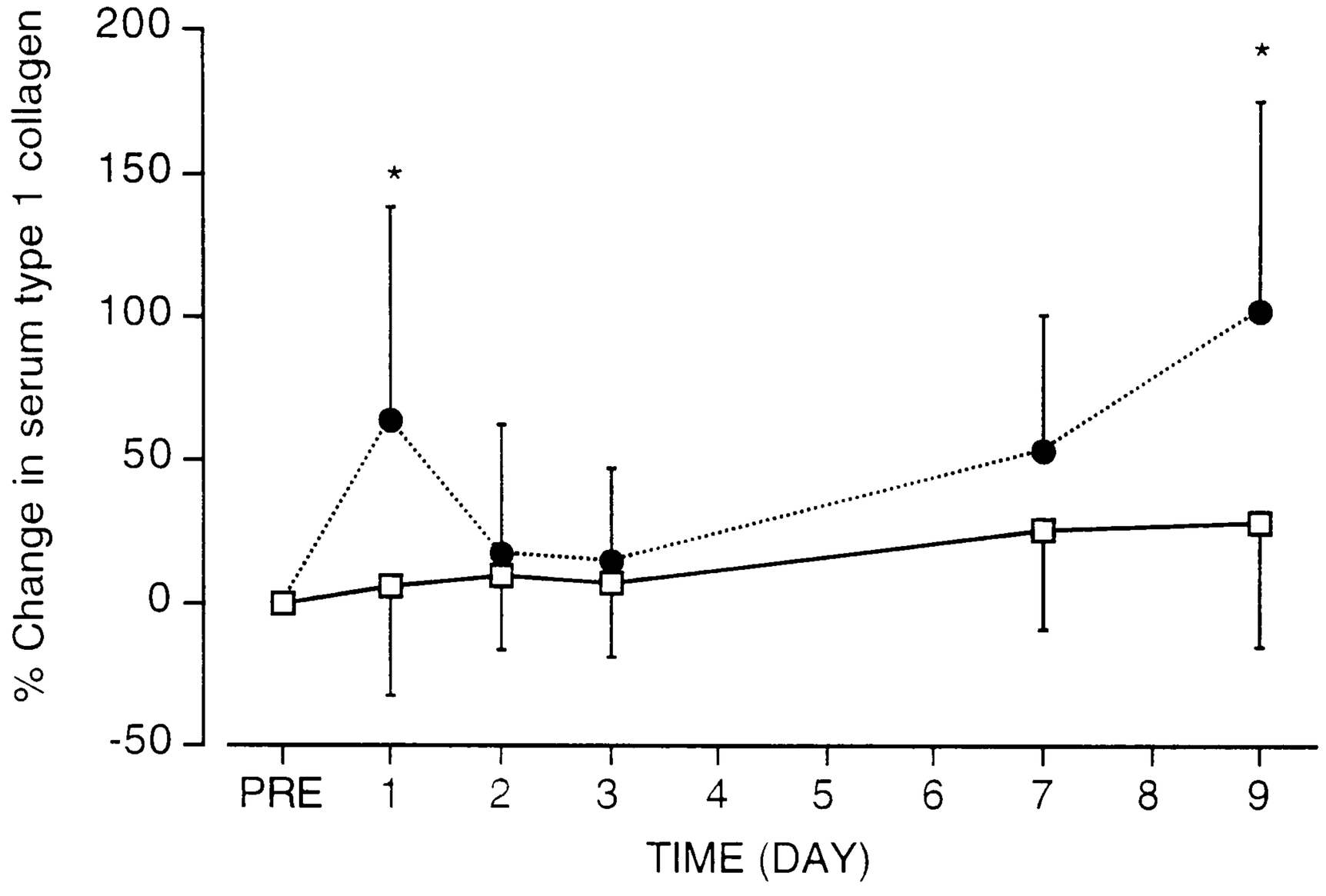

Fig. 5.2.% change in serum type 1 collagen following concentric exercise (open squares) and eccentric exercise (closed circles) of the knee extensors of a single leg.Values are means +/- SD, and P values vs. PRE using Duncans post hoc following repeatedmeasures ANOVA (*=P<0.05).

		PRE-EXERCISE	DAY 1	DAY 2	DAY 3	DAY 7	DAY 9
CON.	SERUM TYPE 1 COLLAGEN ($\mu g.l^{-1}$)	137.2 (57.0)	141.8 (62.2)	144.0 (51.0)	138.2 (41.6)	160.6 (45.2)	162.0 (41.8)
	PLASMA HYDROXYPROLINE ($mg.l^{-1}$)	3.1 (0.9)	3.1 (0.8)	2.9 (0.7)	3.2 (0.9)	3.2 (0.8)	2.9 (0.7)
ECC.	SERUM TYPE 1 COLLAGEN ($\mu g.l^{-1}$)	103.8 (32.0)	154.2 (47.8)	117.8 (49.4)	113.4 (34.6)	149.8 (35.2)	190.2 (21.6)
	PLASMA HYDROXYPROLINE ($mg.l^{-1}$)	3.1 (0.9)	3.5 (1.8)	3.3 (1.2)	3.4 (0.9)	3.1 (0.5)	3.2 (0.7)

Table 5.2. Human serum type 1 collagen and plasma hydroxyproline concentration following concentric and eccentric exercise of the knee extensors of a single leg. Values are means +/- SD. (CON.= concentric, ECC.= eccentric).

Indices of collagen breakdown are shown in table 5.2. Serum levels of type 1 collagen did not change significantly following the concentric exercise bout, although significant % changes above pre-eccentric exercise were recorded following the eccentric bout (Fig. 5.2.). A mean increase of 50.4 +/- 47.8 $\mu g.l^{-1}$ was recorded on day 1 post eccentric exercise, and increases of 46 $\mu g.l^{-1}$ and 86.4 $\mu g.l^{-1}$ were recorded on day 7 and 9 post eccentric exercise respectively (day 1 and day 9 % change above pre-exercsie, $P<0.05$, Duncan post hoc following repeated measures ANOVA). Plasma HP values did not change significantly following either exercise bout, although marginally higher mean values were recorded in the days following eccentric exercise compared to concentric exercise.

5.5. Discussion.

Unaccustomed eccentric exercise, but not concentric exercise, resulted in DOMS, a decrease in isometric muscle force, and increased serum activities of enzymes located in skeletal muscle (e.g. CK). Changes in these indices have been used as indirect evidence of exercise-induced skeletal muscle damage. It has been previously reported (Jones et al, 1986; Schwane and Armstrong, 1983) that an eccentric component in exercise causes muscle injury, and data presented here support these findings.

Serum CK activity is commonly used as an indicator of muscle fibre injury (see 1.3.4.). Other enzymes e.g. lactate dehydrogenase, have also been used to indicate injury to skeletal muscle (Schwane et al, 1983; Nosaka et al, 1992). In the present study, LDH-1 (an iso-enzyme of lactate dehydrogenase) may indicate damage to type 1 fibres or cardiac muscle, since LDH-1 is primarily located in these tissues. It has been shown that type 2 fibres are selectively damaged following eccentric exercise in human muscle (Friden et al, 1983; Jones et al, 1986), a finding similarly reported following animal experimentation (Lieber and Friden, 1988; Lieber et al, 1991). However, other authors (Armstrong et al, 1983; Takala et al, 1989; Virtanen et al, 1993) have suggested that both type 1 and 2 fibres may be damaged during exercise. Therefore, the increased LDH-1 reported following eccentric exercise in the present study may indicate that both type 1 and type 2 fibres are injured using the present exercise protocol.

Indices of connective tissue breakdown showed no changes following concentric exercise, whereas some evidence of increased collagen

breakdown was recorded following the eccentric exercise bout. Both exercise bouts appeared to cause no changes in serum ALP activity, suggesting that osteoblastic activity was not contributing to changes in the systemic concentrations of either type 1 collagen or HP. Collagen breakdown leads to the systemic release of HP, and increased levels of plasma HP have been used clinically as an index of collagen degradation and as a means to identify populations at risk from connective tissue injury (Murguia et al, 1988).

Although higher values of serum HP (approximately 9.0 $\mu g.ml^{-1}$) were reported by Virtanen et al (1993) compared to the plasma HP values reported in the present study, these authors also reported no changes in this non-specific marker of collagen breakdown for up to 4 days post exercise. The method for plasma HP measurement used in the present study incorporated a deproteinising step (see 5.3.4.), and it may be that the higher values reported in serum are reflecting the presence of non-collagenous protein bound HP. Also, circulating free proline interferes with the formation of the Ehrlick chromogen such that artifically high HP values are recorded if proline contaminates the assay. Mean plasma HP values of 4.02 $\mu g.ml^{-1}$ were reported for subjects with connective tissue injuries (Murguia et al, 1988), although values as low as 3.3 $\mu g.ml^{-1}$ were thought to represent a threshold for potential injury risk. Values reported in the present study were consistently below this threshold following concentric exercise, whereas following eccentric exercise, mean values were above this threshold for the first 3 days post exercise. Although speculative, this may suggest that the eccentric bout has induced some breakdown to collagen such that subjects were potentialy at risk from connective tissue injury.

In the present study, no significant changes in plasma HP concentration were recorded following either exercise bout, although a slight increase in plasma HP was observed in the days following eccentric exercise. This may reflect that any exercise-induced changes in plasma HP induced by the present exercise protocols were not detectable, possibly due to normal daily collagen metabolism in all connective tissues, especially bone. Plasma HP is a reflection of whole body collagen catabolism and is not specific for any collagen sub-type or tissue location. Thus, the relatively minor contribution that muscle collagen breakdown has towards total plasma HP concentration may be such that changes in muscle collagen with exercise are not distinguishable. Therefore, the use of plasma HP may be limited as an indicator of exercise-induced musculo-tendinous connective tissue breakdown.

The present study has reported pre-exercise serum type 1 collagen concentrations of 120.5 +/- 44.5 $\mu g.l^{-1}$ for healthy, untrained volunteers, using an ELISA method based on the antigenicity of circulating type 1 collagen presented to a human type 1 collagen specific antibody (Rennard et al 1980). However, the specificity of the binding site has not been established. Similar values for serum type 1 collagen have been reported by previous authors using a radioactive labelled immunoassay (Hartmann et al, 1990), where reference values for healthy adults ranged from 140 - 180 $\mu g.l^{-1}$. Higher values for a mixed male and female group were reported by Saxton and Donnelly (1994), where pre-exercise baseline values were approximately 400 $\mu g.l^{-1}$. Values reported in urine (using a commercially available immunoassay) have ranged from 250 +/- 110 $mg.mol^{-1}$ creatinine for healthy pre-menopausal females, to 416 +/- 189 $mg.mol^{-1}$ creatinine for healthy post menopausal females (Bonde et al, 1994).

Circulating levels of type 1 collagen arise from the breakdown of collagen, as newly synthesised collagen circulates as procollagen. On deposition of new collagen, terminal globular domains (the amino- and carboxyl- terminal propeptides) are cleaved from procollagen, and these enter the systemic circulation. Contaminating collagen synthesis bi-products may interfere with some assays for serum type 1 collagen, thereby contributing to the variation in the reported values. It is proposed that the method used in the present study mainly detected breakdown products due to the specificity of antibody used, and the similar results presented by Hartmann et al (1990) using a radio-immunoassay.

Results form the present study suggest an increase in type 1 collagen breakdown 24 hours following eccentric exercise. Virtanen et al (1993) suggested that a single bout of exercise may cause an initial decrease, and then an increase in the tissue production of type 1 collagen two days after high intensity concentric exercise. Collectively, these results may represent an increase in collagen turnover, such that overall muscle collagen concentration remains stable. It has been reported that collagen accumulation in mouse skeletal muscle injured during exercise occurred only in relation to lethal muscle fibre injuries, whereas sub-lethal injuries did not lead to an increase in total muscle collagen content (Myllyla et al, 1986). Rat skeletal muscle collagen content has been shown to increase with immobilisation (Lapier et al, 1995), and following a 4 week period of forced lengthening contractions (Stauber et al, 1996). However, a single bout of exhaustive running did not produce significant increases in the concentration of muscle hydroxyproline (Karpakka et al, 1990). Thus it

appears that the response of muscle connective tissue to different types of exercise is not fully determined.

The present study has reported indirect evidence of skeletal muscle damage following eccentric but not concentric exercise of the knee extensors. Also, eccentric exercise appeared to cause some increase in the breakdown of collagen, and these increases were not apparent following concentric exercise. However, indirect indices of collagen breakdown should be used with some caution when attempting to establish the occurrence of exercise-induced connective tissue injury.

Chapter 6.

Indices of skeletal muscle damage and connective tissue breakdown following eccentric muscle contractions.

Parts of this study have been published as conference proceedings, and have been published as a full paper:

S.J. Brown, R.B. Child, S.H. Day, and A.E. Donnelly (1996) Human skeletal muscle damage and indirect indices of collagen breakdown. Proceedings of the 16th Alternative Muscle Conference, Wolverhampton University.

S.J. Brown, R.B. Child, S.H. Day, and A.E. Donnelly (1996) Effect of eccentric exercise on human skeletal muscle damage and indices of collagen breakdown. Journal of Physiology 495, 141P.

S.J. Brown, R.B. Child, S.H. Day, and A.E. Donnelly (1997) Indices of skeletal muscle damage and connective tissue breakdown following eccentric muscle contractions. European Journal of Applied Physiology and Occupational Physiology 75, 369-374.

6.1. Abstract.

Indirect indices of exercise-induced human skeletal muscle damage and connective tissue breakdown were studied following a single bout of voluntary eccentric muscle contractions. Subjects (6 female, 2 male), mean (+/-SD) age 22 years (+/-2) performed a bout of 50 maximum voluntary eccentric contractions of the knee extensors of a single leg. The eccentric exercise protocol induced muscle soreness ($P<0.05$ Wilcoxon test), chronic force loss, and a decline in the 20:100 Hz percutaneous electrical myostimulation force ratio ($P<0.01$, repeated measures ANOVA). Serum creatine kinase (CK) and lactate dehydrogenase (LDH) activities were elevated ($P<0.01$, repeated measures ANOVA) following the bout. Mean (+/-SD) CK and LDH recorded 3 days post exercise was 2815 IU. L^{-1} (+/- 4144) and 375 IU. L^{-1} (+/- 198) respectively. Serum alkaline phosphatase activity (ALP) showed no changes throughout the study, and a non-significant increase ($P=0.058$, repeated measures ANOVA) in pyridinoline (PYD) was recorded following the bout. Urinary hydroxyproline (HP) and hydroxylysine (HL) excretion, expressed in terms of creatinine (Cr) concentration, increased after exercise ($P<0.05$ and $P<0.01$ respectively, repeated measures ANOVA). Increased HP:Cr was recorded 2 days post exercise, and HL:Cr was increased above baseline on days 2, 5, and 9 post exercise. Indirect evidence of exercise-induced muscle damage suggested that myofibre disruption was caused by the eccentric contractions. Elevated urine concentrations of indirect indices of collagen breakdown following eccentric exercise suggested an increased breakdown of connective tissue, possibly due to a localised inflammatory response.

6.2. Introduction.

Progressive myofibril degeneration observed in some fibres following unaccustomed eccentric exercise (Jones et al. 1986) may indicate a secondary sequence of events (see 1.2.), with release of intracellular proteins and infiltration of the tissue by neutrophils and macrophages (Round et al. 1987; Kuipers et al. 1983). Inflammatory mediators at the injury site e.g. Interleukin-1 (Baracos et al. 1983; Evans et al. 1986) may stimulate muscle proteolysis, contributing to this secondary degeneration (see 1.2.3.).

The high forces associated with maximum effort eccentric muscle contractions may increase the susceptibility of connective tissue structures to exercise-induced injury (see 1.4.). Delayed onset muscle soreness (DOMS)

may be due to exercise-induced inflammation of muscle connective tissue (Jones and Round, 1990), and a relationship between DOMS and urine hydroxyproline concentration has been reported (Abraham, 1977). However, some criticisms of the work reported by Abraham (1977) have been previously discussed (see 1.4.1.). Direct assessment of eccentric exercise-induced damage to muscle connective tissue has been reported in humans (Stauber et al, 1990). These authors suggested that DOMS was caused by inflammation in response to extracellular matrix disruption, although they acknowledged the limitations of examining biopsy material obtained only at the time of peak soreness. Chronic eccentric training-induced changes to muscle connective tissue have been reported in rats (Stauber et al, 1994), and an increased amount of muscle connective tissue in rat muscle has been shown to protect that muscle from contraction-induced injury (Lapier et al, 1995). At present, it is not clear whether changes in connective tissue architecture (e.g. increased or decreased collagen cross-linking) are a part of the post-eccentric exercise-induced muscle damage process.

Measurement of biochemical markers of collagen breakdown may be used to identify the effect of eccentric exercise on connective tissue (see 1.4.1.). Hydroxyproline (HP) and hydroxylysine (HL) are imino acids characteristic of, but not exclusive to, collagen. Increased levels of HP and HL in the urine have been linked with connective tissue breakdown following exercise (Abraham, 1977; Wheat et al, 1989), although the cause of this breakdown has not been established. Cross-linking of collagen molecules (see 1.4.2.) occurs in the extracellular compartment and involves the oxidation of lysine and hydroxylysine residues by lysyl oxidase. The formation of non-reducible cross-links in mature collagen [e.g. pyridinoline (PYD)] confers stability to collagen fibrils, and cross-links released during collagen remodelling are excreted in the urine. The concentration of pyridinoline and deoxypyridinoline in urine have been used to monitor bone resorption (Ubelhart et al, 1990; Robins et al, 1991), although the effect of exercise on this marker has not been extensively studied.

The aim of this study was to investigate the effect of a single bout of maximum voluntary eccentric exercise on indirect indices of skeletal muscle damage and indirect biochemical markers of collagen breakdown in urine (HP, HL, and PYD). It was hypothesised that both skeletal muscle and muscle connective tissue structures may be disrupted by unaccustomed eccentric exercise.

6.3. Methods.

6.3.1. Subjects.

Eight volunteers, 6 female and 2 male, aged 22 +/-2 years (mean +/- SD), and mass 69 +/-11 kg, signed consent documentation and attended the laboratory for a single familiarisation session. Volunteers had refrained from weight training activities for at least 6 months prior to the study, and Wolverhampton University Ethics Committee approval was obtained for this study.

6.3.2. Exercise bout.

Volunteers performed a single bout of 50 maximum voluntary eccentric muscle contractions using the knee extensors of a single leg. Volunteers performed the exercise while lying in a prone position on an isokinetic dynamometer (Kin-Com, Chattecx, TN., USA). Range of motion at the knee during the bout was 1.75 rad., and angular velocity was fixed at 1.05 rad.s^{-1}. Volunteers were required to perform a maximum isometric contraction against the dynamometer lever arm for approximately 1 s prior to the initiation of the eccentric contraction. With continued maximum effort, subjects were required to resist the forced lengthening of their knee extensors. Throughout the isometric and eccentric muscle contraction period, subjects were verbally encouraged and the force produced was continuously displayed to indicate the strength of contraction. Each eccentric contraction lasted approximately 1.6 s, with a 10 s recovery period between each contraction. Subjects relaxed their knee extensors at the end of the eccentric contraction, and during the recovery phase the relaxed leg was returned to the starting position by the experimenter.

6.3.3. Force Data Collection.

Measures of muscle function were obtained from each subject using previously described methods (see 2.2.). Measures of muscle contractile function in the exercised knee extensors were collected in duplicate and in a randomised order before the exercise bout, following the exercise bout, and on days 1, 2, 3, 7 and 9 following the bout. These measures consisted of :
1. knee extension maximum isometric voluntary contraction force at a knee flexion angle of approximately 1.57 rad. (MVC),

2. the mean tetanic force produced during a 1 s pulse of 100 Hz percutaneous electrical myostimulation (PES),
3. the mean tetanic force produced during a 1 s pulse of 20 Hz PES.
Measures 2 and 3 were used to calculate the 20:100 Hz force ratio, and this ratio was used as an index of low-frequency fatigue (see 1.3.3.). PES was applied to the knee extensor muscles using previously described techniques (see 2.2.2.).

6.3.4. Other indices of muscle damage.

Delayed onset muscle soreness (DOMS) of the exercised knee extensors was assessed daily using a questionnaire incorporating 6 sites on the anterior muscles of the upper leg (see 2.1.).

A 10 ml venous blood sample was collected from the antecubital fossa pre exercise, and on days 1,2,3,7 and 9 following the bout. These time points were chosen in an attempt to indicate any 'short term' responses, i.e. within the initial 72 hours post exercise, and more 'long term' responses on days 7 and 9. Due to subject tolerance and ethical constraints, sampling on all 9 days post exercise was not possible. Serum samples obtained from venous blood (see 2.3.) were stored at -20 degrees C prior to measurement (in duplicate) of creatine kinase (CK) activity (see 2.4.), and lactate dehydrogenase (LDH) activity (see 2.5.).

6.3.5. Indices of connective tissue breakdown.

Alkaline phosphatase (ALP) activity in serum was measured using an assay kit (see 2.6.). A 20 ml mid-flow sample of subjects' first morning urine was collected before exercise, and on days 1, 2, 3, 5, 7, and 9 following the bout. Urine concentration of the collagen cross-link pyridinoline (PYD) was determined by high pressure liquid chromatography (see 2.10.2.). The urine concentration of hydroxyproline (HP) was determined using a spectrophotometric method (see 2.8.1.), and urine hydroxylysine concentration (HL) was determined using previously described methods (2.8.2.). These indices were expressed in relation to the urine concentration of creatinine which was measured using a commercially available assay kit (see 2.7.). All urine samples were analysed in duplicate and in a randomised order.

6.3.6. Statistical analysis.

Soreness data were analysed using Wilcoxon matched pairs tests. Parametric data were analysed using repeated measures analysis of variance (ANOVA) with post hoc analysis using a Duncan's multiple range test. Data for serum CK activity were analysed following logarithmic transformation due to the lack of homogeneity in variance with actual measures of CK activity throughout the time course of the study. Values reported are means +/- SD, and the accepted level of significance was $P<0.05$.

6.4. Results.

All subjects reported soreness in the exercised knee extensors in the days following the bout. In some subjects, the sensation of soreness remained for up to 7 days following the bout (Table 6.1). Chronic force loss of the knee extensors was recorded on the days post exercise (Fig. 6.1), and MVC remained significantly below pre-exercise levels for up to 3 days after the exercise bout ($P<0.01$, Duncan post hoc following repeated measures ANOVA). Although a trend of further declines in force from post exercise to day 3 were observed, MVC on day 3 was not significantly different from MVC post exercise. The 20:100 Hz force ratio was at a minimum following the bout ($P<0.01$ vs pre-exercise, Duncan post hoc following repeated measures ANOVA) and remained lower for a further 24 hours. Thereafter, all values for the 20:100 Hz force ratio were not significantly different from pre-exercise. Pre-exercise serum enzyme activities were within the normal range for all subjects (Table 6.1). Serum CK activity increased following the bout ($P<0.05$, repeated measures ANOVA on log(CK) values). On days 3 and 7 post exercise, serum CK activity was significantly increased ($P<0.05$ vs pre-exercise, Duncan post hoc following repeated measures ANOVA), although sampling between these time points was not carried out. Increased LDH activity in serum was recorded on day 3 post exercise ($P<0.05$ vs pre-exercise, Duncan post hoc following repeated measures ANOVA), and no significant differences were recorded throughout the study for serum ALP activity. Urine levels of Cr showed no changes throughout the study ($P>0.05$ repeated measures ANOVA), therefore HP, HL, and PYD concentrations have been expressed in relation to urine Cr concentration (Fig. 6.2). Urine HP:Cr was at a maximum on day 2 post exercise ($P<0.05$ vs pre-exercise, Duncan post hoc following repeated measures ANOVA) with a 96 % (+/- 117

Table 6.1. Delayed onset muscle soreness (DOMS) in arbitrary units, and serum enzyme activities of creatine kinase (CK), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP), following a single bout of 50 voluntary eccentric contractions of the knee extensors of a single leg.

DOMS		PRE-EXERCISE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
median (range)		6 (6-6)	22a (10-27)	20a (11-40)	18a (8-36)	15a (6-25)	12a (6-19)	9a (6-19)	6 (6-9)	6 (6-6)	---
Serum enzyme activity	CK	42 (16)	464 (719)	747 (773)	2815b (4144)	---	---	---	1563b (1820)	---	436 (428)
mean (+/- SD)	LDH	207 (97)	206 (92)	234 (83)	375c (198)	---	---	---	268 (102)	---	217 (78)
$IU.l^{-1}$	ALP	124 (29)	125 (40)	126 (27)	134 (34)	---	---	---	130 (30)	---	129 (30)

a= P<0.05 vs Pre (Wilcoxon test),
b= P<0.05 above Pre using logged values (Duncan's post hoc following repeated measures ANOVA),
c= P<0.05 above Pre (Duncan's post hoc following repeated measures ANOVA).

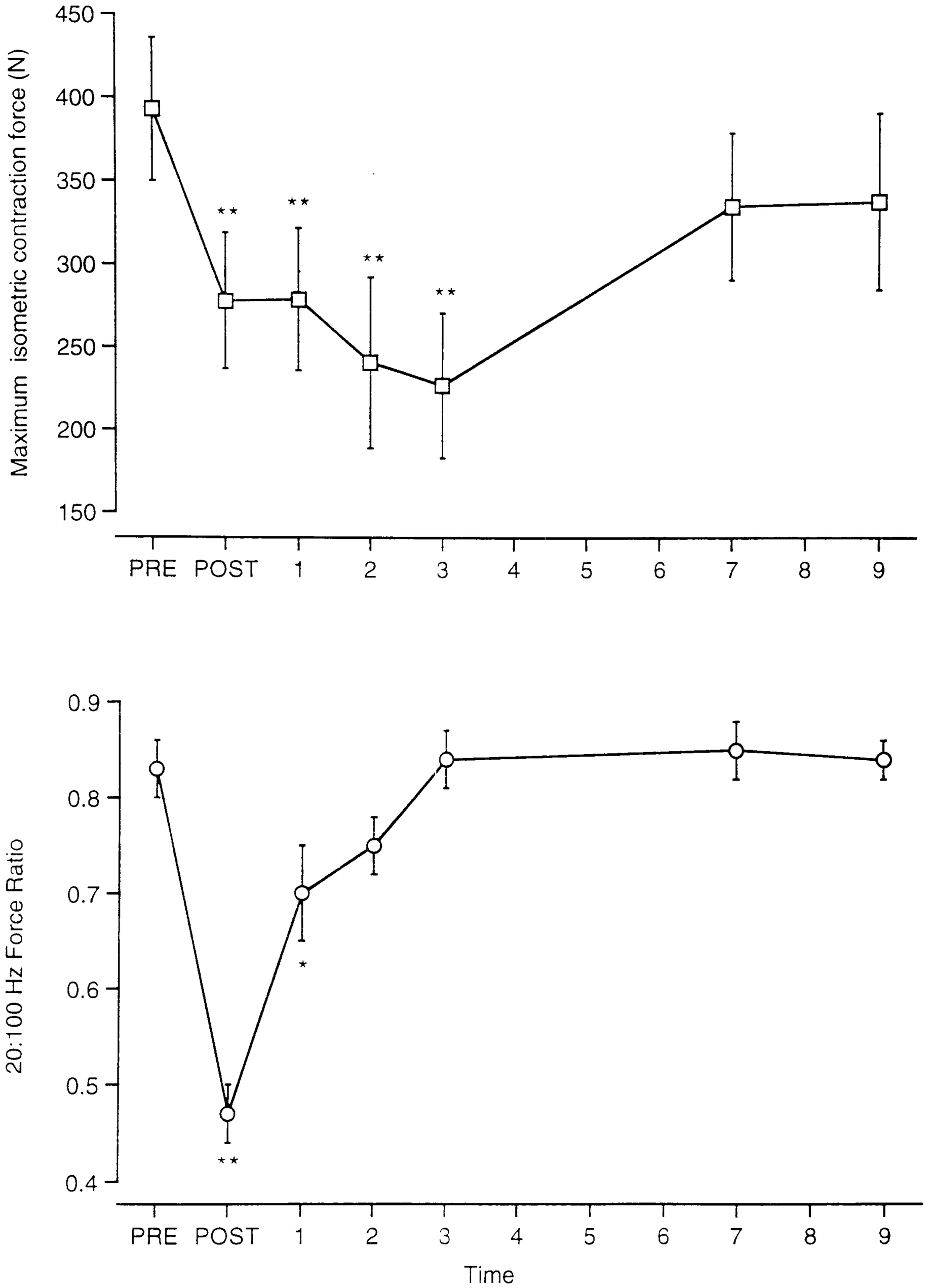

Fig. 6.1. Maximum isometric contraction force (MVC) of the knee extensors (UPPER), and 20:100 Hz stimulated force ratio of the knee extensors (LOWER) following 50 eccentric contractions. Values are means +/- SD. ** = P<0.01, * = P<0.05 vs. PRE, Duncans post hoc following repeated measures ANOVA.

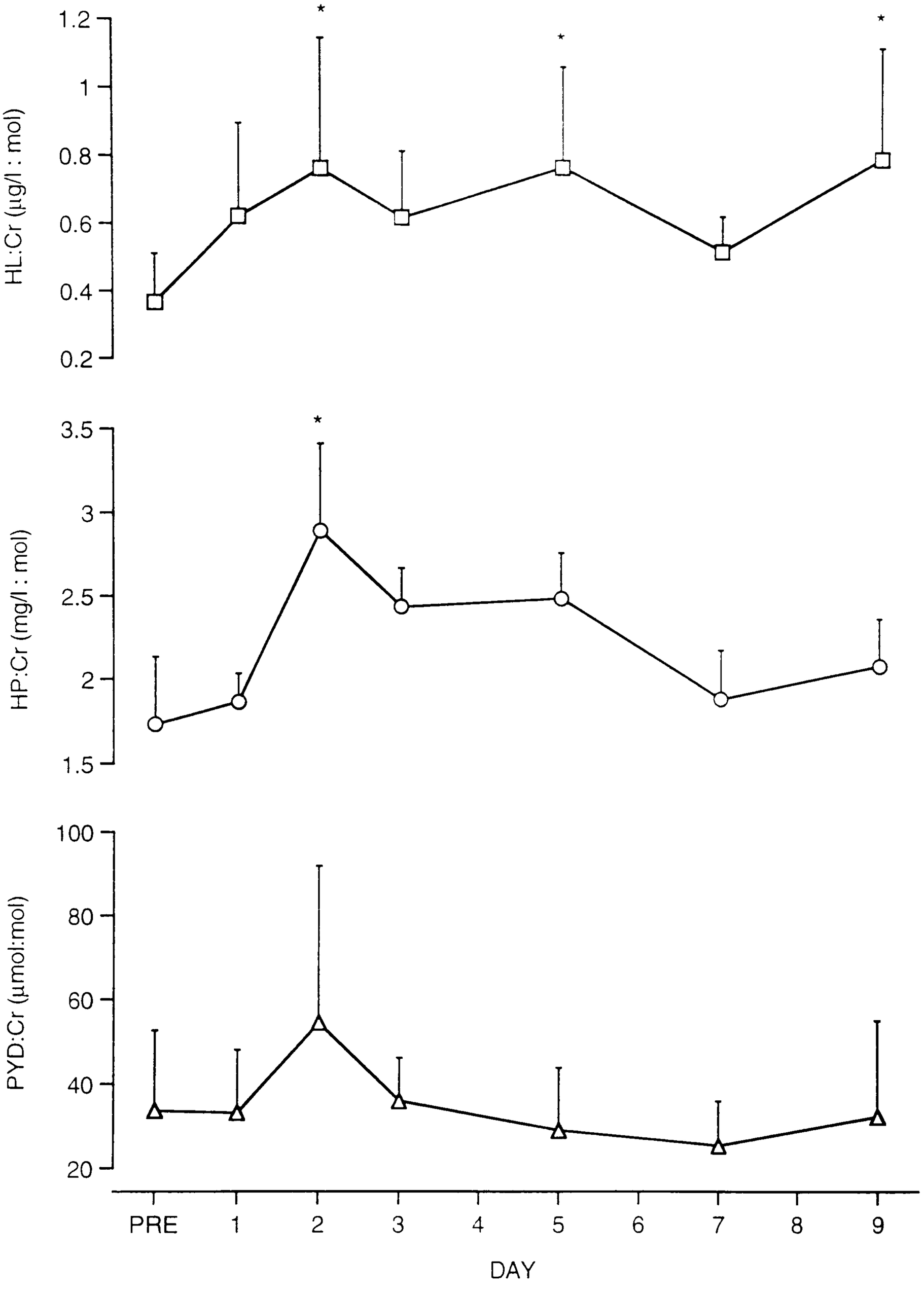

Fig 6.2. Urine indices of collagen breakdown following a single bout of 50 voluntary eccentric contractions of the knee extensors of a single leg (Mean +/- SD). HP=Hydroxyproline; HL=Hydroxylysine; PYD=Pyridinoline; Cr=Creatinine. * = $P<0.05$ vs PRE, Duncan's post hoc following repeated measures ANOVA.

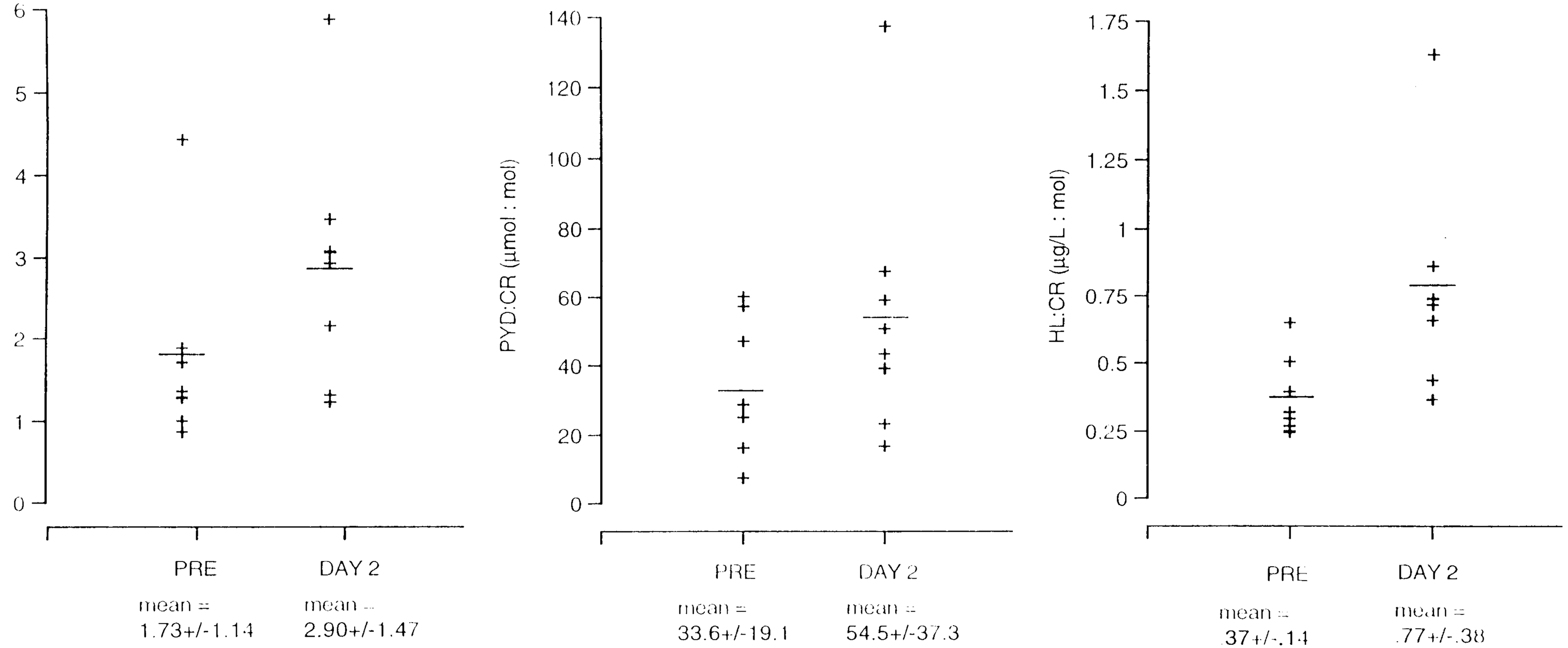

Fig. 6.3. Urinary indices of collagen breakdown pre-exercise and 2 days post exercise. Exercise involved 50 maximum voluntary eccentric repetitions of the knee extensors of a single leg. n=8.

%) increase above pre-exercise values. Urine HL:Cr increased 69 % (+/- 50 %) above pre-exercise on day 2 post-exercise, and was significantly elevated above baseline again on days 5 and 9 post exercise (all $P<0.05$ vs pre-exercise, Duncan post hoc following repeated measures ANOVA). Although outside the accepted level of significance, evidence of a trend in PYD excretion following the bout is presented in Fig. 6.2, where the difference over time for the PYD:Cr ratio was $P=0.058$ (repeated measures ANOVA). Before exercise, mean (+/- SD) urine PYD concentration was 33.6 +/- 19.1 μmol/mol Cr, and highest recorded concentrations of PYD in urine were recorded 2 days after the exercise bout (54.5 +/- 37.4 μmol/mol Cr). Indices of collagen breakdown in the urine pre-exercise and 2 days post exercise are shown in more detail in Fig. 6.3, where each subject's data is shown for these time points.

6.5. Discussion.

Muscle soreness, force loss, low-frequency fatigue, and leakage of muscle enzymes into the circulation are characteristic phenomena associated with unaccustomed exercise, especially types of activity involving eccentric muscle contractions. The present study used these indices as indirect evidence of skeletal muscle damage following a single bout of maximum voluntary eccentric contractions of the knee extensors. This study also used indirect markers of collagen breakdown (HP, HL, and PYD) to examine the effect of eccentric contractions on connective tissue disruption. In the present study, the high force eccentric exercise model has been used to study the damage and repair processes associated with certain types of muscle injury. Maximum voluntary eccentric exercise protocols have been used by previous authors (e.g. Newham et al, 1987), and may represent a useful laboratory method for inducing temporary repairable muscle injury. However, isokinetic eccentric muscle contractions through a large range of motion are not common in human movement, although eccentric muscle actions are a feature of many sporting activities. Therefore the results of the present study should be viewed with caution when considering exercise-induced muscle injury and connective tissue injury in more general terms.

Force loss post exercise may reflect the combined effects of muscle fatigue and muscle damage. Force loss on subsequent days may reflect increasing structural damage observed by previous authors using eccentric exercise protocols (Friden et al, 1983; Jones et al, 1986), and may be contributing to the prolonged force deficit observed in the present study. It

has been suggested (Newham et al, 1987) that force loss and release of intramuscular constituents following eccentric exercise represented the removal of irreparably damaged fibres that may have been particularly susceptible to damage. Increased serum CK activity has previously been used as an indirect marker of muscle membrane permeability changes, and as evidence of injury to muscle fibres (Friden et al, 1989). In the present study, the delayed increase in serum CK and LDH activity possibly indicated delayed skeletal muscle damage attributable to the eccentric exercise bout, however, since these indices were not measured between days 3 and 7 post exercise, it may be that peak activities of CK and LDH in serum were not detected.

The absence of dietary restriction on volunteers during this study may have contributed to the residual variability in urinary indices of collagen breakdown. Hydroxyproline and hydroxylysine may be affected by diet to a greater extent than pyridinoline, although the minimal effect of dietary collagen and gelatin on excretion of hydroxylysine has been reported (Askenasi, 1975). However, since the mean urine hydroxyproline, hydroxylysine, and pyridinoline concentrations all increased post-exercise, and no changes were observed in urinary creatinine levels throughout the study, the present study suggested that the exercise bout (and not diet) caused these increased levels.

Increased circulation during physical activity may enhance clearance of connective tissue resorption products from existing pools, although an exercise-induced wash-out of bone metabolites has been discounted (Kristofferson et al, 1995). In the present study, the delayed increase in markers of collagen breakdown contradict the existence of a connective tissue metabolite reservoir. If an exercise-induced clearance caused immediate breakdown and/or removal of indices of collagen breakdown in urine, the increases would probably be evident shortly after the exercise bout (within 24 hours post-exercise). Similarly, if connective tissue injury resulted directly from mechanical damage incurred during the bout, urinary indices of collagen breakdown would probably increase within 24 hours.

In the present study, the site of collagen breakdown cannot be elucidated. However, since serum ALP activity appeared unaltered following exercise (suggesting no increase in osteoblast activity), it is possible that connective tissue within the muscle and/or tendon was affected by eccentric exercise rather than bone. Degradation and synthesis of new collagen appeared altered following muscle injury (Myllyla et al, 1986) and during exercise (Kovanen and Suominen, 1989). Strain on the musculoskeletal

system induced by high force concentric exercise (Virtanen et al, 1993) appeared to activate type 1 collagen synthesis, and in the present study, high force eccentric contractions appeared to increase the breakdown of collagen. It is not clear whether increased collagen breakdown is balanced with increased collagen synthesis, and the influence of different types of exercise on this balance needs further work.

High forces during eccentric muscle contractions may affect collagen metabolism within the muscle and tendon connective tissue structures. Increased collagen turnover may represent an adaptive response to muscle loading, and increased excretion of HP, HL, and PYD in the urine may indicate the breakdown of collagen as a result of this increased turnover. Connective tissue remodelling may involve transient periods of mechanical weakness (Zamora and Marini, 1988) and structures could be damaged if insufficient time is allowed for adaptation. It has also been suggested that new collagen synthesised in response to exercise lacked mature cross linking (Curwin et al, 1988), and lower levels of pyridinoline in new collagen may decrease its tensile strength. However, these studies used animal models and care should be taken when extrapolating this data to human work.

In the present study, increased indices of collagen breakdown in urine two days after the exercise bout possibly result from an exercise-induced inflammatory response. Exercise-induced muscle injury has been associated with inflammation in muscle connective tissue (Tullson and Armstrong, 1981), and inflammatory mediators may promote collagen breakdown via stimulation of collagenase production in connective tissue synthesising cells. Migration of neutrophils to the injury site and subsequent release of elastase, collagenases, and cytotoxic factors, may possibly induce breakdown of surrounding connective tissue. The macrophage response observed in injured muscle has been characterised by an infiltration of ED1+ and Ia+ macrophage sub populations, and later by an accumulation of ED2+ cells (Stauber et al, 1988; St.Pierre and Tidball, 1994). ED1+ and Ia+ cells predominate in the early stages of muscle degeneration, and in the present study, possibly caused a breakdown of muscle connective tissue. Delayed margination of macrophages in eccentric exercise-induced damaged muscle (Jones et al, 1986; Round et al, 1987), particularly the ED2+ sub population, may be indicative of regeneration, such that collagen synthesis is promoted and the extra cellular matrix is stabilised.

This study has reported indirect evidence of exercise-induced skeletal muscle damage and collagen breakdown following a single bout of 50 eccentric muscle contractions of the knee extensors of a single leg. Indirect indices of collagen breakdown in urine were elevated two days post exercise, and serum CK and LDH activities were elevated three days post exercise. However, from the present data, the mechanisms causing these delayed responses can not be identified.

Chapter 7.

General discussion.

In the preceding chapters, the effects of unaccustomed exercise, particularly eccentric muscle contractions, have been studied. Exercise-induced damage to skeletal muscle has been assessed using indices of muscle function, muscle soreness, and serum enzyme activities. Also, assessment of connective tissue damage following exercise has used indices of collagen breakdown in serum, plasma, and urine.

7.1. Delayed onset muscle soreness (DOMS).

The mechanisms which produce the sensation of pain and soreness in eccentric exercised muscle are not well understood. Estimations of muscle soreness can be subjective, although the assessment of soreness in the studies presented in this thesis remains important. "How sore does your muscle feel?" is an important question which can be used only in human experimentation, and the association between the sensations of pain and other indices of skeletal muscle damage is interesting.

The time course of soreness does not appear to coincide with other indices of exercise induced muscle damage. For example, in chapter 3, soreness was back to baseline values on day 9 post exercise, although MVC and MVS measures were still below pre-exercise values. Also, in chapter 4, serum CK activity was increased up to 7 days post exercise following 50 voluntary eccentric repetitions although soreness was back to baseline by day 7. It is possible that sensory afferent neurones associated with transmitting the sensation of soreness from exercise induced damaged muscle become de-sensitised throughout the damage/recovery process. Alternatively, a delayed yet transient intramuscular oedema (Crenshaw et al, 1994) may provide a mechanical stimulus for the activation of pressure sensitive afferent neurones (Hayward et al, 1991), thus leading to the sensation of DOMS.

It was suggested in chapter 4 (see 4.5.1.) that DOMS may result from a combination of mechanisms, including inflammatory mediators and connective tissue breakdown products. Damage to contractile proteins may contribute to the initial sensations of pain, however it is unlikely that this damage contributed to the DOMS experienced on days when the MVC had returned to pre-exercise values. Pain following eccentric exercise-induced muscle damage may result from connective tissue injury rather than from injury to the active muscle fibres. This was originally proposed by Hough (1902), who suggested that a failure to co-ordinate motor unit innervation during muscular contraction may rupture connective tissue attachment sites,

thus initiating the phenomenon of soreness. Later, Stauber et al (1990a) suggested that DOMS was mediated by the disruption of connective tissue in the extracellular matrix, and not myofiber injury, although data presented in this thesis suggested that the time course of collagen breakdown was not the same as that of DOMS. Pain receptors located in muscle connective tissue may be sensitised in response to connective tissue damage (Stauber, 1989), and it is possible that this sensitisation lasts for longer than any localised elevation of collagen breakdown products. Reported elevations in the urine HP concentration preceded soreness (Abraham, 1977), and this possibly suggested that DOMS may have a role in the protection of damaged connective tissue. However, in chapter 5, increased PYD and HP were recorded on day 2 post exercise, while soreness was elevated on days 1 - 6. These time courses suggested that even if soreness and connective tissue breakdown are closely associated, other factors probably contribute to DOMS. Although attenuated, DOMS continued to be present following a repeated bout (see chapter 4), however it is not known whether this was accompanied by connective tissue breakdown. Further work on repeated bouts of eccentric exercise and subsequent effects on collagen metabolism is needed.

7.2. MVC, MVS, and 20:100 Hz stimulated force ratio.

Changes in selected indices of skeletal muscle function have been measured throughout this thesis, and used as evidence of exercise-induced muscle damage. Indices of muscle function may be informative about muscle performance as a whole (see 1.3.2.), and throughout this thesis, changes in isometric contraction force and muscle force frequency characteristics have been used to indicate the presence of muscle damage.

Force loss appeared not to be a feature of concentric exercise (see chapter 5). Force loss occurring during the days post eccentric exercise in what could be termed "recovery" days, possibly indicated secondary damage initiated by mechanical trauma induced by the bout. Although a minimum MVC has been reported immediately post exercise (Newham et al, 1987; Clarkson et al, 1992), the further declines observed in the MVC in chapters 3, 4, and 6 are similar to those reported in animal experimentation (McCully and Faulkner, 1985; Lieber et al, 1994), and more recently in human work (MacIntyre et al, 1995). This continued decline may reflect the increasing ultrastructural damage observed in biopsy material (Friden et al, 1983; Jones et al, 1986). An initial mechanical event (see 1.1.) may initiate a

degeneration cascade (see 1.2.), and the further declines in MVC during the days post exercise may be reflecting these phenomena.

Eccentric exercise-induced changes in the muscle force-frequency characteristics have been reported in the preceding chapters. Following unaccustomed eccentric exercise, there appeared to be a disproportionate loss of tetanic force at low frequencies of stimulation (e.g. 20 Hz) compared to higher frequencies (e.g. 100 Hz). This has been reported previously (e.g. Newham et al, 1987), although the exact mechanisms of low-frequency fatigue remain uncertain. It has been suggested that the disproportionate loss of force at low frequencies of stimulation may reflect a reduction in the quantity of Ca^{2+} released from the SR per action potential, or a change in the affinity of the troponin binding site for calcium (Jones, 1981). Due to the prolonged time course of recovery from low-frequency fatigue (Edwards et al, 1977), structural disturbances to the SR and T tubules were preferred explanations (Jones, 1981), and work by Westerblad et al (1993) has further demonstrated that reduced Ca^{2+} release from the SR is a phenomena of low-frequency fatigue in amphibian muscle. If low-frequency fatigue is indicative of impaired calcium release, the re-uptake of calcium from the myoplasm during relaxation, as evidenced by muscle relaxation rates presented in chapter 3, appeared unaltered. Thus, a preferred site of low-frequency fatigue may be the T tubule, or damage to one of the proteins involved in E-C coupling, such that at low stimulation frequencies, impaired Ca^{2+} release from the SR is disrupted by processes other than structural damage to the SR.

A relationship between muscle length and force-frequency characteristics has been demonstrated in human skeletal muscle (Sacco and Jones, 1994), such that at low stimulation frequencies, a disproportionate loss of force has been observed at a short muscle length. An 'electrically silent' involuntary shortening of eccentric exercised skeletal muscle has been reported (Clarkson et al, 1992), although the time course of this shortening does not parallel that of low-frequency fatigue. However, Jones (1996) speculated that a damaged muscle may contain over-extended sarcomeres, and as a consequence, the remaining functional sarcomeres may be shorter in a damaged muscle compared to an undamaged condition. Thus, a shift in the force-frequency curve to the right (as in low-frequency fatigue) may be indicative of a redistribution of sarcomere lengths (Jones, 1996), possibly induced by unaccustomed eccentric exercise.

The different time course between the changes in maximum voluntary contraction force (MVC) and the 20:100 Hz stimulated force ratio may indicate that these two functional indices are not of common origin. These indices may be identifying different mechanisms involved in the damage-regeneration process (Sacco and Jones, 1992), and although speculative, their temporal dissociation may indicate a 'prioritised approach' to the repair of exercise-induced muscle damage. MVC represents effective cross-bridge cycling in all recruited, viable motor units. Therefore, a decline in MVC may reflect failure of cross-bridge formation, possibly indicating damage to the contractile proteins. The delayed recovery of MVC (e.g. up to 9 days post exercise in the 50 repetition group, chapter 4) may suggest that the contractile machinery may be extensively damaged. The 20:100 Hz stimulated force ratio is generally at a minimum immediately post exercise, possibly suggesting that this is a function of mechanical damage induced during the bout. The 20:100 Hz stimulated force ratio may represent SR functioning (Jones, 1981), thus SR integrity appears restored within approximately 2 days post exercise. This may indicate that repair of the SR either takes priority during the early stages of recovery, or is not affected by secondary degeneration processes. The primary function of the SR is to regulate intracellular [Ca^{2+}], and close control of this may be essential for muscle regeneration. Elevated intramuscular [Ca^{2+}] and concomitant activation of proteolytic enzymes has been proposed (see 1.2.1.) as a possible mechanism by which secondary degeneration processes cause damage to muscle fibres. A strategy in which priority is given to the repair of the SR over repair of the contractile apparatus may be beneficial in attempting to control a Ca^{2+} activated degeneration cascade. The possible resistance of the SR to damage was indicated in chapter 3 by no change in the muscle relaxation rates (suggesting that the active uptake of intracellular Ca^{2+} by the SR was not affected by eccentric muscle contraction), although the 20:100 Hz data presented in chapter 4 indicated that SR function was as likely to be impaired following a second bout as it was following an initial bout. Functioning of the SR following exercise-induced muscle damage, in both untrained and trained ("protected") muscle requires further research.

In chapter 3, an increase in muscle isometric contraction force was induced by the application of percutaneous electrical myostimulation (PES) to the eccentric exercised knee extensors (described as the MVS technique). Previous authors (Jones et al, 1989b) have reported that no additional force can be obtained using superimposed electrical stimulation on voluntary contractions, although some increases in force could be induced by

superimposed stimulation on contractions at 50 % MVC. Although PES was superimposed during MVC contractions in other studies, the results were equivocal. This may be a possible reflection of the magnitude of injury induced by an electrically stimulated exercise bout compared to a voluntary protocol. It was suggested in chapter 3 that a central limiting mechanism may exist which restricted the recruitment of partially damaged or vulnerable motor units. This was proposed on the basis that the MVS technique consistently increased isometric contraction force by approximately 10 % in eccentric exercised muscle, and not in the pre-exercise condition. Following unaccustomed eccentric exercise, afferent neural feedback from partially damaged or vulnerable muscle fibres in a motor unit may prevent that unit contributing to overall force production. Further analysis of the mechanism by which force loss occurs following eccentric exercise, and the activation patterns of exercise-induced damaged muscle requires more research.

7.3. Electro-mechanical delay (EMD) and connective tissue damage.

A main finding in chapter 3 was an increase in the time delay between the start of PES and the onset of contraction, and this was discussed in terms of K^+ accumulation in T tubules. Electromechanical delay (EMD) may include several events associated with excitation-contraction (E-C) coupling, e.g. conduction of the action potential in the T tubule, release of Ca^{2+} from the SR, and subsequent formation of the cross bridge. However, Komi (1984) reported that the primary cause of EMD was likely to be stretching of the series elastic component in the muscle. Therefore, another possible mechanism for the increased EMD reported in chapter 3 may be damage to connective tissue structures in the series elastic component.

By attaching muscles to other structures (e.g. bone) via relatively compliant tendons, the operating range of the myotendinous unit increases. However, the use of a compliant force transmission structure introduces a delay between the time when the muscle develops tension and the production of movement (Lieber, 1991). An exercise-induced decrease in tendon stiffness (i.e. increased compliance), possibly due to the breakdown of intermolecular cross-links in collagen fibres (reported in chapter 6), may have the effect of delaying the effective transmission of force produced during muscle contraction. This may be the cause of the increased EMD on day 3 post exercise (chapter 3), although elevation of the urine concentration of collagen cross links was recorded 48 hours post exercise (chapter 6). This

temporal discrepancy may be explained by the use of different exercise models used to induce skeletal muscle damage. More extensive damage may result from stimulated eccentric exercise, as indicated indirectly by higher CK values, greater loss of MVC, and more soreness. Thus, using the stimulated exercise model, the full extent of collagen breakdown and changes in connective tissue compliance may not be manifest until 72 hours post exercise.

Changes in connective tissue compliance do not explain why the increased EMD was significant with 100 Hz PES and not with 20 Hz PES. Again, in chapter 3 this was discussed in terms of K^+ accumulation, with particular reference to the critical level of 10 mM in T tubules induced by two impulses at 100 Hz (see 3.5.). It has been suggested that an undamaged tendon is stiffer at higher loads (e.g. tetanic force produced during 100 Hz PES) (Komi, 1984), and therefore elongates relatively less than when subjected to lower loads, when the tendon is more compliant (e.g. tetanic force produced by 20 Hz PES). This would imply that the delay should be longer at lower frequencies of stimulation when lower forces are produced. Alternatively, the behaviour of damaged tendons to different loads may not be the same as their undamaged counterparts. It has been suggested that remodelling of connective tissue involves a transient period of weakness (Zamora and Marini, 1988), and this may result in the release of intrinsic tension on collagen fibres as a precursor for reconstruction. Therefore, the increased EMD may be evidence of this transient decrease in connective tissue stiffness, and the suggestion of an increase in PYD excretion only on day 2 post-exercise (chapter 6) may further indicate the transience of this period of weakness. Although speculative, these findings may suggest that a period of time exists during which connective tissue is vulnerable to further injury, and it may be identified by changes in EMD. The possibility of a "window of vulnerability" for connective tissue remodelling needs further research.

7.4. Exercise-induced damage to connective tissue.

High force eccentric exercise was shown to cause an increase in connective tissue breakdown products in urine (chapter 6), plasma (chapter 5), and in serum (chapter 5). Data presented in chapter 5 suggested that increased collagen breakdown, as evidenced by increased serum type 1 collagen antigenicity, may accompany eccentric exercise. Further evidence of eccentric exercise-induced damage to collagen was presented in chapter

6, where increases in PYD, HP, and HL in urine were recorded post exercise. However, the mechanisms responsible for increased collagen breakdown can only be speculated on. Also, the specific site of collagen breakdown can not be determined. In chapters 5 and 6, no change in serum ALP activity possibly indicated that osteoblastic activity was not affected by exercise, suggesting that altered bone metabolism may not be contributing to increases in collagen breakdown.

Exercise-induced inflammation has been linked with DOMS (for reviews, see: Smith, 1991; MacIntyre et al, 1995), and connective tissue disruption during the initial stages of exercise-induced muscle damage may initiate this inflammation. Stauber (1989) suggested that the inflammatory cell margination seen in eccentric exercised muscle (Round et al, 1987; Jones et al, 1986) may be mediated by extracellular matrix degradation products released from disruption of the endomysium. However, cellular infiltration of the muscle has been reported as a response to damage rather than it's cause (Jones et al, 1986). Localised fluctuations in the activity of extracellular matrix degrading metalloproteinases, possibly as a result of eccentric exercise, may contribute to the controlled turnover of extracellular matrix components. However, the effect of eccentric exercise on production of matrix metalloproteinases has not been fully determined, although stromelysin-1 gene transcription was induced by the inflammatory cytokine, interleukin-1 (Quinones et al. 1994). Lysosomal enzymes may also have a role as mediators of inflammation, such that selective secretion of lysosomal contents from marginalised neutrophils may result in the provocation of acute inflammation, and thus to a localised degradation of connective tissue (Ignarro, 1974). Elevated intracellular Ca^{2+} and concomitant increased levels of cyclic guanosine 3, 5, monophosphate (GMP) stimulate the secretion of lysosomal constituents into the extracellular environment. It may be possible that raised intramuscular Ca^{2+} and accompanying increased cyclic GMP may signal release of intramuscular lysosomes into the extracellular matrix, thereby causing some localised connective tissue degeneration. This may represent a link between the theories of delayed increased intracellular $[Ca^{2+}]$ (Armstrong, 1984) due to transient SR damage (Byrd, 1992), and connective tissue breakdown.

Although the mechanisms which bring about connective tissue remodelling remain unclear, there seems to be a need for a mechanism by which mechanical loading of connective tissue structures can induce biological remodelling. Prostaglandin E_2 (PGE_2) may act as a "transducer" between mechanical stress and collagen metabolism as in vitro work on

osteoblasts (Yeh and Rodhan. 1984) indicated a role for prostaglandins in the translation of mechanical stimuli to cellular activity. A localised increase in PGE_2 concentration in exercise-induced damaged muscle (suggested by Armstrong, 1984) may be part of the mechanism by which muscle contraction can influence collagen synthesis. Fibroblasts in connective tissue may produce insulin like growth factor-1 (IGF-1) in response to mechanical stress (Jones and Round, 1990), and an exercise stimulus has been shown to increase the expression of IGF-1 in rat tendons and the surrounding paretenon cells. Since IGF-1 may stimulate new collagen synthesis, this marker potentially represents a link between mechanical demand and biological adaptation. Alternatively, Skerry (1990) proposed that load-related reorientation of proteoglycans (and not collagen fibres) may be the link between mechanical loading and connective tissue remodelling. Further research directed toward elucidating the mechanism of connective tissue remodelling and the influence of different muscle contraction modalities on this remodelling is required.

Curwin et al (1988) reported lower levels of PYD in the tendons of exercised animals compared to non-exercised controls, despite increased collagen deposition in the exercised animal. These authors suggested that tendons of the 'runners' were less mature than those of the control animals, and a possible explanation for this was that increased collagen turnover resulted in increased accumulation of immature collagen. It was suggested (Curwin et al, 1988) that lower levels of mature collagen cross-links may lower the tensile strength of the collagen matrix as a result of an exercise-induced increase in tissue turnover producing a more immature tendon collagen matrix. The increase in PYD 2 days post exercise reported in chapter 6 does not necessarily support the hypothesis of an exercise-induced increase in collagen turnover, although comparison of results from a single bout of exercise used in chapter 5 and an 8 week extensive running protocol (Curwin et al, 1988) may not be valid. Similarly, no increases in plasma HP following either concentric or eccentric exercise may suggest that exercise per se does not influence overall collagen turnover.

In humans, the extent of damage to a specific fibre type following eccentric exercise may be related to the connective tissue associated with that fibre type. Stauber (1989) speculated that type 2 fibres may be more susceptible to eccentric exercise-induced muscle damage due to the difference between type 1 and type 2 fibre endomysium, although experimental evidence of this in humans is limited. Zimmerman et al (1993) reported that for rat skeletal muscle, the soleus muscle (predominantly type 1

fibres) had a higher collagen content than the gastrocnemius muscle (predominantly type 2 fibres), and that this was irrespective of age or training status. These authors reported that exercise appeared to have no effect on collagen content in either muscle type, although age associated increases in muscle [PYD] was more pronounced in the soleus than gastrocnemius muscle. Following limb casting (Lapier et al, 1995), increased muscle connective tissue content increased the muscle's resistance to exercise-induced injury, and this could have been due to selective type 2 fibre atrophy during limb immobilisation. However, the relationship between susceptibility of human skeletal muscle to exercise-induced damage, and any possible accompanying changes in intramuscular connective tissue, requires further research.

7.5. Conclusions and suggested further work.

Studies presented in this thesis have examined indices of muscle damage and connective tissue breakdown following predominantly eccentric muscle contractions. Non-invasive methods of muscle function, and indirect biochemical indices of muscle injury, were used to examine muscle function and muscle adaptation following eccentric exercise. Indirect indices of collagen breakdown have been used as evidence of connective tissue injury following eccentric exercise.

Evidence of collagen breakdown was reported 1 day after eccentric exercise (increased serum type 1 collagen concentration) and 3 days after eccentric exercise (increases in urine PYD, HP, and HL concentration). It has been suggested that changes in these indices of collagen breakdown may reflect eccentric exercise induced damage to connective tissue, possibly due to the initial stages of an exercise-induced inflammatory response.

Suggested further work :

1. Direct analysis of connective tissue changes in human skeletal muscle following eccentric muscle contractions using biopsy material. Tissue may be used to identify changes in connective tissue content (e.g. endomysial thickening) and changes in collagen structure (e.g. cross-link concentration).
2. Identify a relationship between changes in indirect indices of collagen breakdown and changes in muscle tissue.
3. Determine the relationship between force, work, and strain range during muscle lengthening contractions with respect to indices of collagen metabolism.

4. Identify the temporal relationship between indices of collagen breakdown and synthesis following eccentric exercise, and examine this relationship following a variety of exercise protocols e.g. endurance exercise.
5. Complement the markers used in this thesis with indices of elastin breakdown and proteoglycan synthesis in order to examine the effects of exercise on other components of connective tissue.

Chapter 8.

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Appendix 1.

Muscle soreness questionaire.

Soreness Questionnaire

Name.....

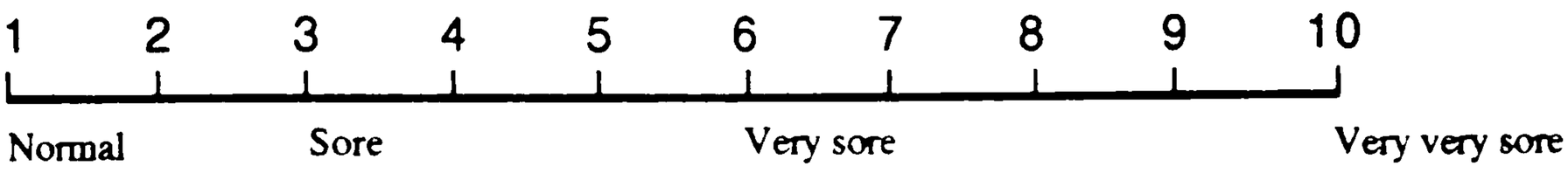
Type of Exercise.....

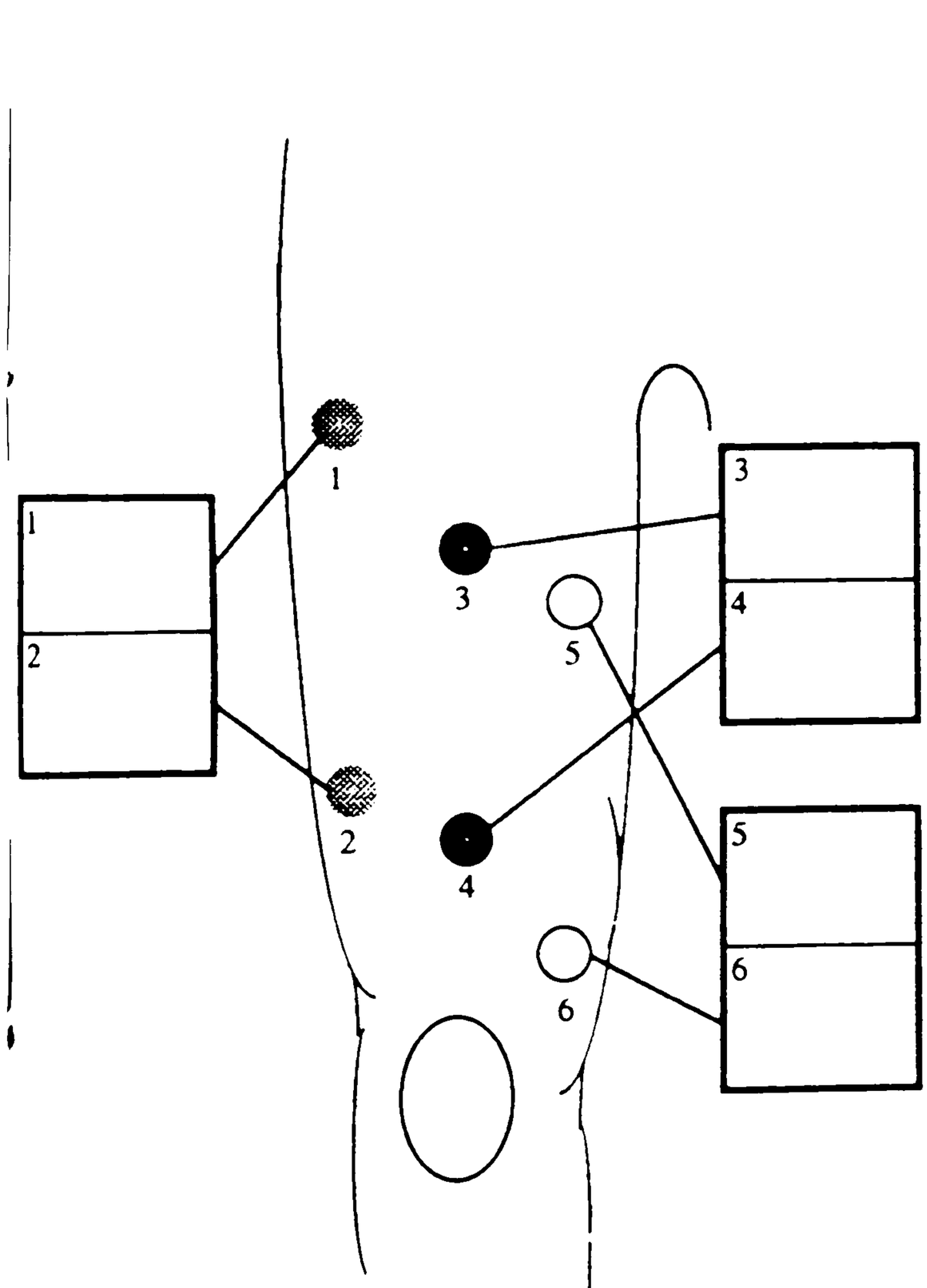
Days Following Exercise.....

Date.....

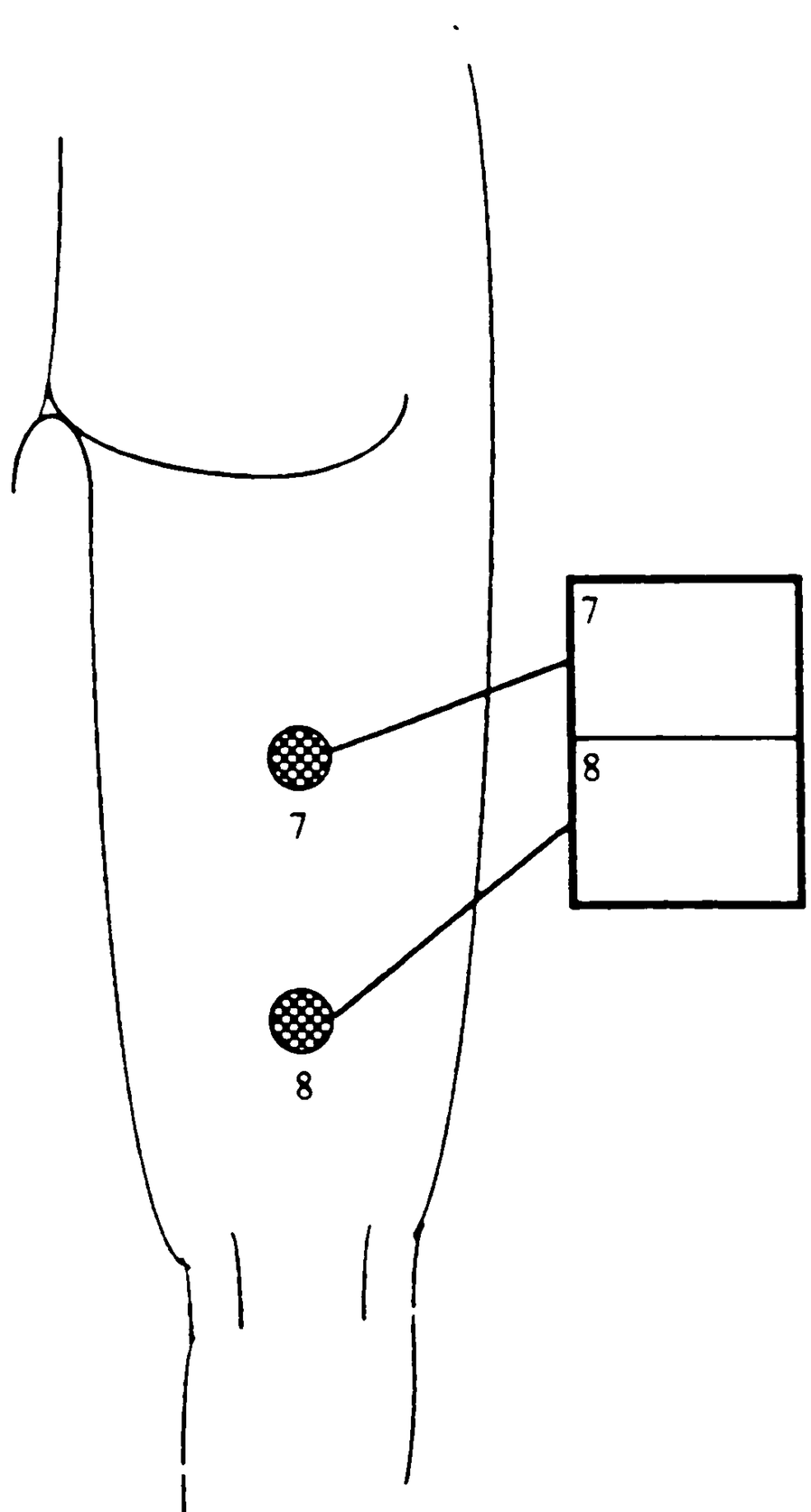
The purpose of this questionnaire is to evaluate muscle soreness post exercise.
Determine the degree of soreness by pressing on the leg muscle at the six sites below

Record the soreness value as a number between 1 and 10 using the soreness scale below as a guide.





FRONT OF RIGHT THIGH



BACK OF RIGHT THIGH

Quads mean soreness	-
VL mean soreness	-
Distal V.L. soreness	-

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